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SUPPLEMENTS

MATERIALS AND METHODS

Animals and sensitization Male albino guinea pigs weighing 350 g to 450 g were sensitized to killed mycobacteria in adjuvant as described previously (11) 7 animals were killed one month and 11 one year later Other guinea pigs were passively sensitized with the immune sera of these animals, by giving 3 times 3 ml into the hind thigh muscles (injection I 27 hours, injection II 19 hours and injection III 3 hours before killing)

Skin testing, migration inhibition technique and haemagglutinating and haemolysing antibody titration were described in previous articles (11, 12) Inactivated sera were absorbed with 1 5 packed sheep red cells for one hour at room temperature —The amount of purified diphtheria toxoid (DT, Lot No *35 1550 Lf/ml 1827 LF/mg N*, Orion Pharmaceutical Co, Helsinki) used to sensitize tanned sheep red cells for antibody titration was 5 mg/1cc packed cells

Measurement of cytophilic antibody The rosette forming techniques described by *Boyden* (5) and *Jonas et al* (10) were used slightly modified

Freshly drawn sheep red blood cells were tanned and sensitized as described by *Sodomann et al* (15) with 10 mg/1cc packed cells of Tuberculin Purified Protein Derivative (PPD Parke Davis et Company Detroit) or with 20 mg/1cc of DT The cells were made as a 2 per cent suspension in phosphate buffer pH 7 2 containing 1 100 normal rabbit serum. To detect antibody cytophilic for macrophages on the surface of peritoneal cells of immunized animals one part of the peritoneal cells for migration inhibition tests was separated washed in Hanks salt solution and twice in phosphate buffer pH 7 2 and suspended at 20×10^4 cells/ml in phosphate buffer pH 7 2 To detect cytophilic antibody in sera 5×10^6 peritoneal exudate cells of normal animals per ml of 1 10 serum dilution in Hanks solution were incubated for one hour at room temperature with occasional shaking The cells were washed once in Hanks solution and divided into two parts One was washed twice in phosphate buffer *pH 7 2 the cells were suspended* at 20×10^4 cells/ml in phosphate buffer pH 7 2 and used for rosettes The other part was washed twice in Hanks solution and used for migration inhibition tests

For Boyden's incubation technique 0 1 ml of peritoneal cell suspension was mixed with 0 8 ml of phosphate buffer pH 7 2 and 0 1 ml of sensitized red cells was added The tubes were incubated at *+4° C overnight* the cells suspended with a Pasteur pipette and those cells with 5 or more erythrocytes on their surface were counted/400 peritoneal cells on glass slides The percentage of rosettes was calculated from this When the centrifugation technique described by Jonas was used 0 1 ml of sensitized red cell suspension was added to 0 1 ml of peritoneal cell suspension The cells were centrifuged at +4° C for 5 min at 900 rpm suspended with a Pasteur pipette and reactions (graded – to + + +) read on glass slides (Fig 1)

RESULTS

I *Actively Immunized Animals*

Skin tests Normal animals had negative skin reactions to 10 μg of PPD One month after immunization the animals had weak 4 hour skin reactions (diameter 8 to 10 mm, induration — to +) and 24 hour skin reactions were positive (12 to 18 mm +) One year after immunization 4 hour skin reactions were stronger (10 to 17 mm + to + +) as were 24 hour reactions (12 to 25 mm, + + + to + + + +)

Migration tests (Fig 2) All antigen concentrations studied (from 0 01 μg PPD/ml to 25 μg PPD/ml) inhibited peritoneal cells taken one year after immunization more than those taken after one month One year after

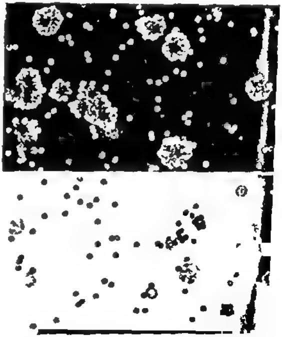


Fig 1 Peritoneal exudate cell rosettes of a guinea pig one year after immunization with killed tubercle bacilli in adjuvant Centrifugation technique tanned sheep red cells coated with PPD + + + reaction (upper) DT — reaction (lower) Magnification × 320

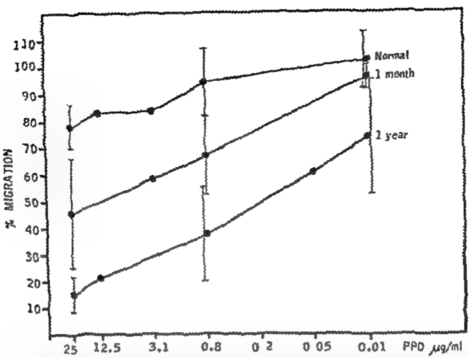


Fig. 2. Per cent migrations of guinea pig peritoneal exudate cells in the presence of various amounts of PPD. Each line is drawn through the average of the experiments made using cells of normal guinea pigs and of those one month or one year after immunization with killed tubercle bacilli in adjuvant. The bars indicate standard deviation.

sensitization 0.01 µg PPD/ml was also inhibitory (t-test, $P<0.05$).

Cytophilic antibody (Fig. 1 and 3). Normal peritoneal exudate cells contained on average 3 per cent rosette forming cells with PPD coated sheep red cells and 2 per cent with DT coated cells. None were detected with the centrifugation technique. One month after immunization the percentages of rosettes were 4 and 1 respectively. In 3 out of 5 animals the centrifugation technique gave a weak (+) reaction with PPD cells. One year after sensitization the peritoneal cells of individual animals formed variable numbers of rosettes with PPD cells (from 6 to 66 per cent, average 27 per cent), but the number of unspecific DT cells had not increased (average 3 per cent). The centrifugation technique gave strongly positive reactions with PPD cells (mostly ++ or +++); using DT cells results were negative.

Haemagglutinating and haemolysing antibody. With PPD coated sheep red cells the passive haemagglutinin titres were equally high one month and one year after immunization. Titres were partly reduced by treatment with 2-mercaptoethanol. Antibody measured by passive immune haemolysis was found only one year after sensitization. All haemolysins disappeared after treatment with mercaptoethanol. Red cells coated with DT gave negative reactions (Table 1).

II. *Normal Peritoneal Cells Treated with Immune Sera*

Migration tests. 1. Pooled normal homologous guinea pig serum was replaced by 15 per cent serum from the normal or immunized animals as final media in migration chambers (Table 2). In the presence of one month immune sera 2 µg PPD/ml specifically inhibited migration (average per cent migration ± standard error 39 ± 9.7, $P<0.01$).

1*

TABLE 1 *Antibodies to PPD Measured by Passive Haemagglutination and Passive Immune Haemolysis in Sera of Guinea Pigs Immunized with Killed Tubercle Bacilli in Adjuvant*

Time after immunization	No of animals	Range of titres (first dilution 1 10) Haemagglutination	Haemolysis
Normal	8	–	–
1 month	7	1 1280–1 5120	–
1 year	11	1 1280–1 5120	1 160–1 2560

PPD-induced inhibition in the presence of one year sera was even more pronounced (average per cent migration 21 ± 3 3, P< 0 001), but also somewhat unspecific because even DT caused some inhibition (average per cent migration 71 ± 8 5, P<0 01)

2 Normal cells were first incubated in a 1 10 dilution of normal or immune sera and the washed cells were studied in migration chambers in the presence of pooled normal homologous guinea pig serum (Table 3) Incubation in one month sera did not inhibit migration in the presence of antigen Incubation in one year sera changed the normal cells so that their migration was inhibited by PPD (average per cent migration 51 ± 5 5 versus 79 ± 3 9, P<0 01) and to some extent also by DT (average per cent migration 74 ± 8 5, P<0 05)

Cytophilic antibody The other part of the normal peritoneal cells incubated in diluted sera was tested for rosette formation (Fig 4) Incubation in normal or one month sera gave rosette counts with PPD cells that were not significantly above background (= non incubated cells), but the centrifugation technique gave some weak positive reactions with one month sera Incubation in one year sera sensitized normal cells (average rosette percentage 32, centrifugation technique reactions from + to + + +) No significant

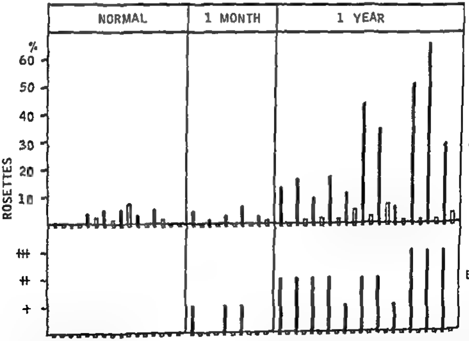


Fig 3 Peritoneal exudate cell rosettes of normal guinea pigs and of animals one month or one year after immunization with killed tubercle bacilli in adjuvant Tanned sheep red cells coated with PPD ■ or DT ▫ Each pair of columns represents one animal tested with two technical modifications A Incubation technique per cent of peritoneal cells with 5 or more erythrocytes on their surface B Centrifugation technique rosette forming reaction graded — to + + +

TABLE ■ *Per cent Migration of Normal Guinea Pig Peritoneal Exudate Cells in the Presence of PPD (25 μg/ml) or DT (30 μg/ml) when 15 Per Cent Normal Serum or Sera from Guinea Pigs One Month or One Year after Immunization with Killed Tubercle Bacilli in Adjuvant Was Used as Final Media in Migration Chambers*

I I	Pooled normal serum		Normal sera		1 month sera		1 year sera	
	PPD	DT	PPD	DT	PPD	DT	PPD	DT
	72 2	99.5	90 8	107 9	ND	ND	21 5	100 9
	92 3	126 1	108 4	99 8	17.5	101 6	19 0	56 3
	94 1	95 2	91 9	121 3	75 4	101 3	31 6	55 4
	88 7	108 3	ND	ND	31 5	103 4	11 2	65 8
					32 2	107 6		
	93 9	101 6	96 1	112 3	40 0	82 9	21 6	78 3
rage SE	88±4 1	106±5 4	97±4 0	110±4 5	39±9 7	99±4 0	21±3 3	71±8 5

ich experiment involves pooled cells of 3 to 5 guinea pigs and individual sera
D = not done

numbers of rosettes were formed with DT coated cells

III *Animals Sensitized Passively with Sera (Table 4)*

Migration tests The intramuscular injection of immune sera into normal animals did not change significantly the average percentage migration of their peritoneal cells Both PPD and DT inhibited slightly the migration of peritoneal cells of some of the guinea pigs that had been injected with one year immune sera

Cytophilic antibody Most one year immune sera made the peritoneal cells of normal animals give weak rosette reactions with PPD cells

Haemagglutinating and haemolysing antibody After passive sensitization with immune sera almost all animals had detectable antibody in their sera

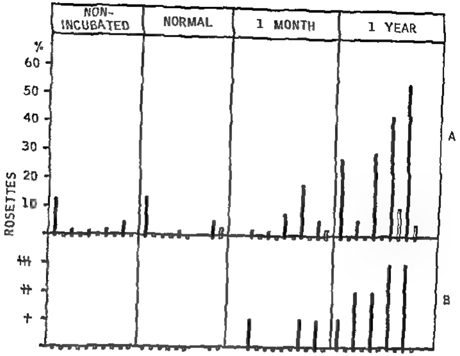
DISCUSSION

When guinea pigs are sensitized with killed tubercle bacilli in adjuvant, they develop

TABLE 3 *Per Cent Migration of Normal Guinea Pig Peritoneal Exudate Cells in the Presence of PPD (25 μg/ml) or DT (30 μg/ml) after Incubation in 1 10 Dilution of Normal Serum or Sera from Guinea Pigs One Month or One Year after Immunization with Killed Tubercle Bacilli in Adjuvant*

Cell pool No	Normal sera		1 month sera		1 year sera	
	PPD	DT	PPD	DT	PPD	DT
1	77 0	81 7	ND	ND	49 9	87 8
2	79 4	118 4	67 7	102 8	57 4	82 7
3	69 8	96 8	97 3	101 0	53 9	70 7
4	ND	ND	82 7	90 7	30 7	42 4
			70 6	88 2		
5	88 7	108 6	80 7	92 8	63 2	86 5
Average ± SE	79±3 9	101±7 9	80±5 2	95±2 9	51±5 5	74±8 5

Each experiment involves pooled cells of 3 to 5 guinea pigs and individual sera
ND not done



two technical modifications A Incubation technique per cent of peritoneal cells with 5 or more erythrocytes on their surface B Centrifugation technique rosette forming reaction graded — to + + +

sensitivity to tuberculin as measured by skin reactivity, peritoneal cell migration inhibition and serum antibody titrations (11) The sensitivity of the animals is greatest 4–6 months and one year after sensitization (11 12) The present results show that one year after such immunization cytophilic anti-PPD antibody could be found on the surface of peritoneal exudate macrophages (Fig 3) and also in the serum (Fig 4) One month after immunization small amounts of cytophilic antibody were found Haemolysing anti-PPD antibody too was only detectable late after immunization (Table 1), but it was sensitive to treatment with 2 mercaptoethanol, whereas with other antigens cytophilic antibody has been shown to be 7S (3, 9)

David et al (6) were unable to inhibit the migration of normal peritoneal cells with immune sera and antigens However, *Bloom and Bennett* (4) and *Amos et al* (1), who gave PPD boosters to guinea pigs immunized with tubercle bacilli, had positive results with the sera of these animals and PPD The latter (1) demonstrated by the rosette forming technique that peritoneal macrophages incubated in these sera had cytophilic antibody on their surface *Heise et al* (8) incubated normal guinea pig alveolar macrophages in immune sera and showed that their migration was inhibited in the presence of PPD They also could elute the antibody from the surface of macrophages According to *Spitler et al* (16) human red cells sensitized with IgG antibody link adjacent macrophages together and thus produce migration inhibition Apparently macrophages have a receptor for IgG on their surface (3, 9)

In the present experiments PPD plus either one year or one month immune sera inhibited the migration of normal peritoneal cells (Table 2) When the cells were only incubated in sera and washed, one month sera did not inhibit migration (Table 3) *Berken*

and Benacerraf (3) have found that cytophilic antibody complexed with antigen has an increased affinity for macrophages. This could be the reason for the inhibition observed when one month sera were present in the migration chambers together with antigen. One year immune sera contained so much more or more avid cytophilic antibody that they could sensitize cells even in the absence of antigen. The migration of the sensitized cells could be inhibited by PPD and they could be shown to have cytophilic antibody on their surface (Table 3 and Fig. 4). However, an unspecific antigen (DT) also produced some migration inhibition (Table 3). The reason for this might be some factor in immune sera that changes the surface and mobility of macrophages in the presence of different proteins.

When normal guinea pigs were injected in-

TABLE 4. *Peritoneal Exudate Cell Per Cent Migration and Rosettes, and Serum Antibodies Measured by Passive Haemagglutination and Passive Immune Haemolysis in Guinea Pigs Injected Intramuscularly with 9 ml of Normal Sera or Sera from Animals One Month or One Year after Immunization with Killed Tubercle Bacilli in Adjuvant. Rosette Reaction Tested with Incubation (A) and Centrifugation (B) Technique*

	Antigen used							
	PPD					DT		
Sera	Per cent migration (25 μg/ml)	Rosettes A %	Rosettes B	Titre (first dilution 1:10) Haemagglutination	Titre (first dilution 1:10) Haemolysis	Per cent migration (30 μg/ml)	Rosettes A %	Rosettes B
Normal	87.6	2.50	—	—	—	98.2	0	—
	77.9	2.00	—	—	—	97.0	1.75	—
	98.8	6.00	—	—	—	112.7	0.25	—
	57.2	0	—	—	—	105.1	0	—
	ND	3.00	—	—	—	ND	0	—
Average ± SE	80 ± 8.8	3				103 ± 3.6	0.4	
1 month	79.8	11.50	+	1:320	—	94.2	1.00	—
	80.1	1.50	—	—	—	98.7	0.25	—
	80.8	0.75	—	1:320	—	81.9	0.25	—
	62.2	0.25	—	1:640	—	94.0	0.25	—
	79.1	0	—	1:160	—	99.9	0	—
	67.7	0.50	—	1:320	—	111.0	0	—
	ND	1.25	—	1:640	—	ND	0	
Average ± SE	75 ± 3.2	2				97 ± 3.9	0.3	
1 year	66.2	6.00	+	—	1:80	92.3	0	—
	56.3	5.75	+	1:2560	1:80	87.0	0	—
	55.4	14.50	+	1:40	1:80	89.4	0.50	—
	103.0	1.75	—	1:1280	1:40	106.1	0	—
	67.6	1.00	—	1:20	1:160	77.5	0	—
	ND	9.25	+	—	—	ND	0.50	—
	ND	12.75	+	1:20	1:80	ND	0.50	—
Average ± SE	70 ± 8.2	7				91 ± 4.6	0.2	

ND = not done

tramuscularly with one year immune sera, both PPD and DT inhibited slightly the migration of the peritoneal cells of some animals The cells of these animals gave weak rosette reactions with PPD cells (Table 4) Sera of almost all passively sensitized animals showed haemagglutinating and haemolysing antibody activity, but migration inhibition did not correlate with these as *Kosluala* (11) has already found in individual actively immunized animals

Using PPD coated red cells, the numbers of rosettes produced by peritoneal cells, either one year after immunization or after passive sensitization by incubation in one year immune sera, were of the same magnitude (27 per cent and 32 per cent median reaction ++ in both cases) Per cent migration using 25 μg PPD/ml was 15 in actively immunized animals and 51 with cells sensitized passively by incubation However DT also caused some migration inhibition of passively sensitized cells (per cent migration 74) Thus it is impossible to rule out completely a contribution by serum factors on the surface of macrophages to the strong migration inhibition reactions seen one year after active immunization with killed tubercle bacilli However, in animals immunized with DT so as to have large amounts of cytophilic antibodies but negative 24 hour skin reactions, migration inhibition is negative (13)

According to *Siskind and Benacerraf* (14) as the antigen concentration falls after immunization cells with receptors of progressively higher affinities proliferate Thus the average affinity of circulating antibodies increases The findings of *Bast et al* (2) suggest a similar process during the development of delayed reactivity to human serum albumin (HSA) Stimulation of thymidine incorporation in lymph node cell cultures of guinea pigs was induced with progressively lower concentrations of HSA until 4 weeks after sensitization in Freund s complete adjuvant This was better correlated with delayed hypersensitivity than with antibody titres According to the present results and those of the earlier work of one of us (12) the production of MIF can also be induced with decreasing concentrations of PPD during the course of immunization The reason for the very prolonged process of sensitization could be the fact that the animals are sensitized with whole tubercle bacilli in adjuvant The liberation of antigen from these deposits is rather prolonged Because the production of MIF is thought to correspond well to delayed reactivity, the production of it with progressively lower concentrations of antigen at later times favours increasing affinity of lymphocytes for the antigen in the evolution of cellular immunity also

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ENHANCED SUSCEPTIBILITY TO INFECTION

A New Method for the Evaluation of Neutrophil Granulocyte Functions

CLAUS O SOLBERG

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A new method for the evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes in human disease has been developed by which the total number of viable bacteria and the number of viable intracellular bacteria in an *in vitro* phagocytic system are measured Standardization of the granulocyte, bacteria and serum opsonin concentrations at their optimum has made available a sensitive method by which minor variations in neutrophil granulocyte functions can be measured In a preliminary clinical study patients with unrecognized abnormalities of neutrophil granulocyte functions were readily diagnosed The results indicate that abnormal neutrophil granulocyte function is more prevalent than realized

Phagocytosis and intracellular killing of bacteria are important physiological functions of neutrophil granulocytes, and several disease syndromes have been related to defects in either of these functions (6, 7, 9, 11, 12, 13) Diagnosis of these disease syndromes requires analysis of both phagocytic and bactericidal activities of the granulocytes (6, 13) However, many of the studies on interactions of bacteria and granulocytes have been confined to the ingestion phase or phagocytosis and little attention has been paid to the dynamics of the intracellular phase (for review see 3, 8) Difficulties of methodology have also resulted in divergent interpretations of experimental results A major problem has remained the separation of extracellular and intracellular bacteria in an *in vitro* phagocytic system in order to evaluate and compare the phagocytic and the intracellular bactericidal processes (5, 16)

In a previous study (15), it was demonstrated that phenylbutazone effectively prevented killing of phagocytized bacteria by the granulocytes and that intracellular bacteria were protected from the antibacterial effect of penicillin G and streptomycin even in concentrations that killed more than 98 per cent of extracellular organisms in less than 15 minutes Based on these findings a new method for the evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes was described, using high concentrations of antibiotics for the inactivation of extracellular bacteria and phenylbutazone for the inhibition of intracellular killing of bacteria (15) This study presents the results of standardization of the method in the presence of factors known to influence the phagocytic and bactericidal activities of neutrophil granulocytes Finally patients with diseases related to defects in neutrophil granulocyte functions are described

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MATERIALS AND METHODS

Leucocytes

Heparinized venous blood (10 units of heparin per ml of blood) was obtained from patients and normal individuals and layered on top of a two phase cell separation system in Falcon disposable plastic tubes (16×150 mm) The cell separation mixture contained 10 parts Isopaque (Natrii N-methyl 3 5-diacetamido-2, 4, 6-trijodbenzoas) 33 9 per cent (obtained by dilution with distilled water of Isopaque 75 per cent manufactured by Nyegaard & Co Oslo, Norway) and 20 parts dextran [redacted] per cent (obtained by dilution with distilled water of Dextran 500 provided by Pharmacia, Uppsala, Sweden) Volumes giving blood columns of 40 to 50 mm were employed, and the volume of Isopaque dextran mixture was ¾ of the blood volume When the erythrocytes had passed the interface between plasma and Isopaque dextran, the leucocyte rich plasma layer was pipetted off and centrifuged at 500 [redacted] for 5 minutes The cellular pellet was washed twice in 5 ml heparinized saline (1 unit heparin per ml saline) by centrifugation at 500 g for 5 minutes After the final centrifugation a differential count was performed and the cells were resuspended in Hank's balanced salt solution containing 0 1 per cent gelatin to make appropriate concentrations of neutrophil granulocytes (usually 1×10^7 neutrophil granulocytes per ml) Ninety five to 99 per cent of the isolated leucocytes resisted staining with trypan blue, and their functional integrity was intact as measured by latex particle phagocytosis Erythrocyte contamination in 50 consecutive specimens varied from 14 to 62 per cent (mean 41 per cent) eosinophil granulocyte contamination from 1 to 11 per cent (mean 5 per cent), basophil granulocyte contamination from 0 to 2 per cent (mean 0 8 per cent), and finally lymphocyte monocyte contamination varied from 10 to 23 per cent (mean 15 per cent))

Serum

Pooled fresh normal human serum from 8 adults was stored in 1 ml aliquots at −30° C Immediately prior to each experiment 1 ml of freshly thawed serum was diluted in Hank s balanced salt solution containing 0 1 per cent gelatin to make appropriate serum concentrations The experiments in the present study were performed with the same pool of serum

Bacteria

Staphylococcus aureus 'Oxford' ('Heatley' strain —obtained from the National Collection of Type Cultures, Colindale, London 1958) was cultured overnight in Penassay broth (Difco) and washed twice in [redacted]45 per cent saline as previosuly described (15) The bacteria were suspended in Hank's balanced salt solution to an optical density of 0 6 at 620 nm in a Beckman spectrophotometer This suspension was diluted in Hank's balanced salt solution containing 0 1 per cent gelatin to make appropriate bacteria concentrations (usually 8–12×10^7 colony forming units per ml)

Leucocyte Bacteria Suspension

0 5 ml leucocyte suspension, 0 1 ml bacteria suspension, and 0 4 ml diluted serum were added to 12×75 mm disposable plastic tubes This provided approximately 2 bacteria per neutrophil granulocyte and a final concentration of 10 per cent serum if not otherwise stated The tubes were incubated at 37° C with an end over end rotation in an Heto rotomixer (manufactured by Heto, Birkerød, Denmark) to promote contact between bacteria and leucocytes Samples were removed at prescribed intervals for determinations of the total number of viable bacteria and the number of viable intracellular bacteria The bactericidal activity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or the number of viable intracellular bacteria (15) The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (15)

Total Number of Viable Bacteria

The total number of viable bacteria was determined as follows Using a micro pipetting system (manufactured by Oxford Laboratories, San Mateo, California, USA) $^1/_{100}$ ml of the leucocyte bacteria suspension was added to 1 ml distilled water to facilitate osmotic disruption of the leucocytes Quantitation of viable bacteria was made from appropriate dilutions of this suspension utilizing a standard pour plate technique and Penassay agar (Difco)

The Number of Viable Intracellular Bacteria

The number of viable intracellular bacteria was determined as follows $^1/_{100}$ ml of the leucocyte-bacteria suspension and 1 ml Hank s balanced salt solution containing 0 1 per cent gelatin, 500 µg streptomycin, 500 units penicillin G and 2 mg phenylbutazone (manufactured by Geigy, Basel, Switzerland) were added to 12×75 mm disposable plastic tubes incubated at 37° C for 15 minutes, and centrifuged for 10 minutes at 500 g The cellular pellet was washed twice in 5 ml Hank's balanced salt solution by centrifugation at 500 g for 10 minutes and resuspended in 1 ml distilled water to facilitate osmotic disruption of the leucocytes Quantitation of viable bacteria in this final sus-

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A New Method for the Evaluation of Neutrophil Granulocyte Functions

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In a previous study (15), it was demonstrated that phenylbutazone effectively prevented killing of phagocytized bacteria by the granulocytes and that intracellular bacteria were protected from the antibacterial effect of penicillin G and streptomycin even in concentrations that killed more than 98 per cent of extracellular organisms in less than 15 minutes Based on these findings a new method for the evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes was described, using high concentrations of antibiotics for the inactivation of extracellular bacteria and phenylbutazone for the inhibition of intracellular killing of bacteria (15) This study presents the results of standardization of the method in the presence of factors known to influence the phagocytic and bactericidal activities of neutrophil granulocytes Finally, patients with diseases related to defects in neutrophil granulocyte functions are described

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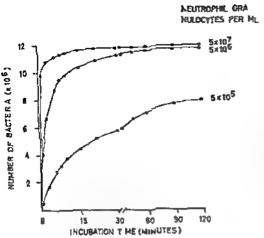

Fig 2 Number of phagocytized bacteria in relation to neutrophil granulocyte concentration (mean of five experiments)

in the leucocyte bacteria suspension was varied by tenfold increments from 5×10^5 to 5×10^7 neutrophils per ml The tests were incubated at 37° C and samples obtained at prescribed intervals Fig 1 gives the total number of viable bacteria and the number of viable intracellular bacteria in the leucocyte bacteria suspensions during incubation, and Fig 2 the number of phagocytized bacteria Phagocytosis and intracellular killing of bacteria increased significantly with the concentration of the phagocytes in the test system Extensive phagocytosis and intracellular killing were demonstrated particularly during the early phase of incubation and relatively small numbers of bacteria were phagocytized and killed after one hour s incubation

Bacterial Concentration

Four tests were performed simultaneously with leucocytes from one person A tenfold dilution of bacteria from 1×10^7 to 1×10^4 was added to individual tubes containing 5×10^6 neutrophils and 10 per cent serum The test tubes were incubated at 37° C and samples obtained for the determination of the total number of viable bacteria and the number of viable intracellular bacteria The results are presented in Tables 1 and 2 as percentages of inocula phagocytized and percentages retaining viability, respectively In relation to the great range of phagocyte/bacteria ratio from 500 1 to 0 5 1, only minor differences in the percentages of inocula phagocytized by the granulocytes and in the percentages of inocula retaining viability were observed From Figs 1 and 2 and Tables 1 and 2 it is evident that the concentration of phagocytes in the leucocyte bacteria suspension is far more important for the phagocytic and bactericidal activities than the phagocyte/bacteria ratio

TABLE 1 *Phagocytosis in Relation to Bacteria Concentration (Mean of Three Experiments)*

No of bacteria in inoculum	Neutrophil/bacteria ratio	Per cent of inoculum phagocytized	
		15 minute sample	120 minute sample
1×10^4	500 1	96 9	99 7
1×10^5	50 1	91 2	98 8
1×10^6	5 1	91 0	98 7
1×10^7	0 5 1	89 4	98 1

5×10^6 neutrophil granulocytes per ml granulocyte bacteria suspension

TABLE 2 *Bacterial Viability in Relation to Bacteria Concentration of the Phagocytic System (Mean of Three Experiments)*

No of bacteria in inoculum	Neutrophil/bacteria ratio	Per cent of inoculum retaining viability	
		15 minute sample	120 minute sample
1×10^4	500 1	5 1	0 9
1×10^5	50 1	10 4	2 0
1×10^6	5 1	10 7	2 1
1×10^7	0 5 1	12 8	2 8

5×10^6 neutrophil granulocytes per ml granulocyte bacteria suspension

Serum Concentration

Three experiments were performed with leucocytes from three healthy individuals For each experiment serum concentrations of 0 2, 5, 10 and 20 per cent were used The neu-

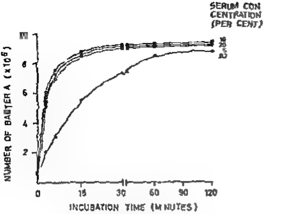

Fig 3 Number of phagocytized bacteria in relation to serum opsonin concentration (mean of three experiments)

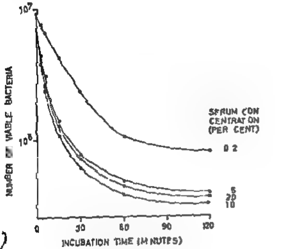

Fig 4 Viable bacterial counts in relation to serum opsonin concentration (mean of three experiments)

trophil granulocyte and bacteria concentrations were kept constant viz 5×10^6 neutrophils and 1×10^7 bacteria per ml leucocyte bacteria suspension The test tubes were incubated at 37° C and samples obtained at prescribed intervals for the determination of the total number of viable bacteria and the number of viable intracellular bacteria Test suspensions containing 5 per cent serum improved phagocytosis (Fig 3) and intracellular killing (Fig 4) significantly compared to test suspensions containing 0 2 per cent serum Ten per cent serum stimulated the phagocytic and bactericidal activities somewhat more than 5 per cent serum However raising the serum concentration to 20 per cent did not further increase phagocytosis and intracellular killing of bacteria

Reproducibility of the Method

Four tests were performed simultaneously with leucocytes from one individual Based on the results of the previous experiments, the following leucocyte, bacteria and serum concentrations were used 5×10^6 neutrophil granulocytes and 1×10^7 bacteria per ml and a final concentration of 10 per cent serum Fig 5 gives the total number of viable bac-

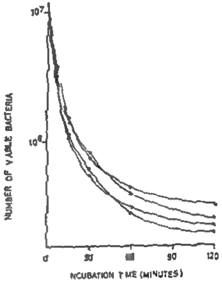

Fig 5 Viable bacterial counts in four identical phagocytic systems

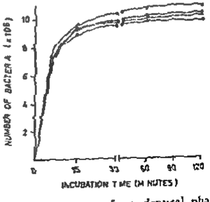

Fig 6 Phagocytosis in four identical phagocytic systems

teria in the leucocyte bacteria suspensions during incubation, and Fig 6 the number of bacteria phagocytized Good agreement between the results of the tests was demonstrated, indicating that the reproducibility of the technique was satisfactory

Preliminary Clinical Studies

So far the test described has been used for the evaluation of neutrophil granulocyte functions in 30 patients with supposedly increased susceptibility to infection Defects in neutrophil granulocyte functions were demonstrated in four patients and two patients with major defects will be described

The diagnosis of fatal granulomatous disease in childhood was established in a 3 year-old boy with chronic pulmonary infection, draining adenopathy, dermatitis and hepatomegaly The results of neutrophil granulocyte function studies in this patient are presented in Fig 7 and by way of comparison, results of studies of neutrophil granulocytes obtained from a normal control In the test sample from the patient, about 50 per cent of the bacteria remained viable after incubation for 120 minutes, and the great majority of the bacteria were located intracellularly, indicating normal phagocytosis, but a defect in intracellular killing of bacteria In contrast, more than 96 per cent of the bacteria incubated with granulocytes from the normal control were killed after incubation for 120 minutes

The second patient was a 64 year-old male who had been treated for [illegible] months with various antibiotics for recurrent enterococcal septicaemia to develop after a transurethral prostatectomy Repeated examinations of the patient and analysis of physiological and immunological factors which might influence the susceptibility to infection had not revealed any abnormalities However, studies of neutrophil granulocyte functions disclosed impaired intracellular killing of bacteria, but a

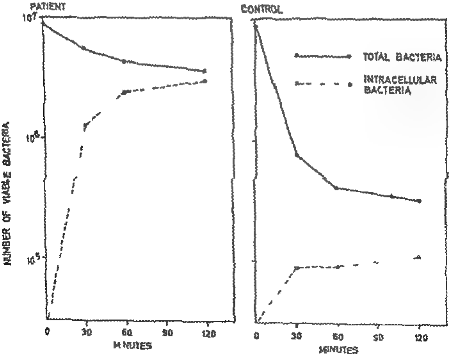

Fig 7 Viable bacterial counts during incubation with neutrophil granulocytes from a patient with fatal granulomatous disease and from a normal control

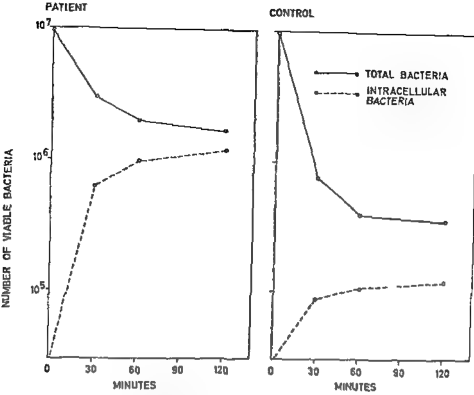

Fig 8 Viable bacterial counts during incubation with neutrophil granulocytes from a patient with long term septicaemia and from a normal control

phagocytic activity close to normal (Fig 8) The patient was treated with large doses of ampicillin and kanamycin for 2 weeks He then became afebrile and the sedimentation rate and peripheral white blod cell count became normal one week later Treatment was, however, continued for another 6 weeks, and repeated evaluation of neutrophil granulocyte functions 2 weeks later revealed no abnormalities The patient has now been in good health for 4 months

DISCUSSION

The reasons why some individuals have increased susceptibility to infections are often poorly understood In bacterial infections phagocytosis and intracellular killing of bacteria by neutrophil granulocytes are essential functions in host defence, and numerous attempts have been made to relate abnormalities in these functions to the development of sepsis However, difficulties of methodology and lack of standardization of techniques have resulted in divergent interpretations of the experimental results

A major problem has remained the elimination of nonphagocytized bacteria in an *in vitro* phagocytic system in order to study the intracellular bactericidal processes (1 2, 5, 16) The original technique of *Maaloe* (10), using differential centrifugation for the separation of extracellular and intracellular bacteria, has been adopted and modified by several investigators (4, 13, 14) These techniques measure cell-associated bacteria and bacteria killed in an *in vitro* phagocytic system, but do not permit separation of phagocytized bacteria from bacteria which have become adherent to the external granulocyte wall Contamination with extracellular bacteria and immune adherence of bacteria to contaminating erythrocytes may also significantly obscure the results (5 16)

To avoid these difficulties some investigators have used antibiotics in the extra

cellular medium to destroy nonphagocytized bacteria. However, interpretation of results obtained in experiments where antibiotics are used may be difficult since differentiation between bacterial inactivation by antibiotics versus granulocytes may be uncertain. As previously demonstrated, inactivation of extracellular bacteria by antibiotics takes about 10–15 minutes (15). During this period, killing of intracellular bacteria by granulocytes may markedly obscure the results (15). Accordingly, a method for the determination of the number of viable intracellular bacteria after a prescribed period of incubation requires not only control of extracellular bacteria but also inhibition of the bactericidal activity of the granulocytes while extracellular inactivation takes place (15). The essence of the present method is, therefore, the combined use of phenylbutazone for the inhibition of intracellular killing of bacteria and high concentrations of antibiotics for the control of extracellular organisms.

The cell separation system and the concentrations of bacteria, granulocytes and serum in the leucocyte-bacteria suspension are by no means original, but standardization of these variables at their optimum and the combined use of phenylbutazone and antibiotics have made available a test system which offers not only a precise evaluation of the phagocytosis, but also of the dynamics of the intracellular phase of bacteria. Our results of preliminary clinical studies indicate that the test may be of particular value in the diagnosis of diseases with various neutrophil granulocyte defects like fatal granulomatous disease of the childhood. Even more important however is the demonstration in our second patient with long term septicaemia of a reversible defect in bactericidal activity of the granulocytes suggesting that the present test may be a valuable tool in the future study of the many clinical circumstances where increased susceptibility to infection remains unexplained by current analysis of host defence mechanisms.

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ALBUMINGLUTAMATE AND ITS USE IN THE PRODUCTION OF PURE CRYSTALLINE ALBUMIN

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Albuminglutamate (Alb glut), the salt of albumin and L-glutamic acid, is formed as a crystalline precipitate when a solution of albumin in half saturated ammonium sulphate solution is acidified with L-glutamic acid lower than the isoelectrical point of albumin, pH 4 7 By acidification down to pH 4 1, the crystallization of albumin as Alb glut is quantitative

Albumin is a simple protein, coagulable by heat, soluble in water and diluted salt solutions, and is precipitated by neutral salts in organic solvents It is symmetrical and represents 50–60 per cent of these proteins It is known to have two main functions in the body viz maintenance of colloid pressure and nutrition of the tissues *White & Weinstein* (*1947*)

The properties of serum albumin to combine with organic ions have been reviewed by *Steinhardt* (1945) It was found by *Boyer* (1945) that caprylate, even at low concentrations protects serum albumin from denaturation in urea or guanidine hydrochloride

Davis & Dubos (1946) found that albumin has affinity for the oleate anion and will remove small amounts of it almost quantitatively from solution a finding which proved to be of importance in the preparation of culture medium for the growth of the tubercle bacillus

Hansen & Holm (1950) found that Foot and Mouth (FM) Disease virus combines with serum albumin, and the biological and physical properties of the FM virus might prove to be different in such a complex

Hansen & Videbæk (1959) showed that Factor IX (Koller) can be extracted from Cohn's Fraction IV by means of an albumin solution to provide a remedy for the treatment of Christmas disease

The present technique to eliminate from protein mixtures the content of albumin was utilized by *Hansen & Clausen* (1964) for production of glucoproteins from serum

The crude Alb glut as obtained from various materials containing albumin such as blood plasma, placental extract, hen ovo—albumen, and others is contaminated with other proteins, lipids and colouring matter It is the author's experience that many of these compounds are bound to the albumin, thus making it necessary to introduce enzyme treatment into the purification process

The present communication describes that the various components of the crude Alb glut are liberated under influence of α amylase, after which it is relatively easy *to produce*

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pure albumin from the Alb glut This may probably be due to the partial elimination of the carbohydrate moiety of albumin

MATERIALS AND METHODS

α-amylase During the experimental work, a standard preparation of swine pancreas α amylase, from Messrs Worthington Biochemical Co, Freehold, N Y, was used From this enzyme, a standard solution in 50 per cent glycerol containing 1200 SOMOGYI units/100 ml, was prepared

The technique elaborated by *Rinderknecht* (1967) was used for estimation of the enzyme activity

Later forced by a growing demand for the enzyme α amylase, a special technique for preparing of the crystalline enzyme was elaborated This preparation has been used in the routine work of our laboratory, since α amylase with the same satisfactory activity as the former product could be prepared from wheat bran Earlier preparations based on similar principles were published by *Helferich* (1935)

As it was found that the enzyme is unstable in dry state it is always kept in solution in 50 per cent glycerol The activity will then remain unaltered for years when stored in the cool

For preparation of α amylase 500 g wheat bran is activated by repeated day long extractions with a mixture of 2700 ml acetone and 900 ml water After five extractions, the material is dried in the air overnight The product is extracted by stirring in 7500 ml water for two hours The extract is clarified by centrifugation and filtration The enzyme is adsorbed from the extract by activated Alhydrogel (the so called Y adsorbent) (vide infra) in a quantity estimated by flocculation titration on a sample

The enzyme is eluted from the adsorbate by suspending in a 20 per cent saturated ammonium sulphate solution which is allowed to stand for 24 hours in the cool after which the supernatant is isolated by centrifugation

The enzyme is crystallized by salting out the concentration of ammonium sulphate being increased up to 0 31 g/cm³, which corresponds the specific gravity 1 176 in the solution (22°C)

After standing for 48 hours at 2° C the crystalline enzyme is obtained by centrifugation and it is immediately dissolved in 100 ml of 50 per cent glycerol

Alhydrogel (Superfos Amaliegade 15 1297 Copenhagen K) is a 2 per cent suspension of $Al(OH)_3$ which *is* delivered with constant adsorption capacity sterile, and without content of pyrogen The adsorbing capacity of Alhydrogel is controlled by measuring its effectivity to adsorb the toxin from a standard diphteria toxin (*Hansen* 1933)

Y adsorbent is Alhydrogel activated especially by means of tetraphenylboron sodium (Kalignost Messrs Heyl & Co) to give an adsorbent useful in the preparation of several crystalline enzymes pure

Vigorous stirring is carried out, during which a solution of 1 g tetraphenylboron sodium in 1 litre distilled water is added slowly Stirring is continued for a further five minutes and then the system is allowed to stand for 30 minutes Centrifugation is carried out (6000 × g) and sufficient distilled water is added to the sediment phase to make a volume of 8 litres, after which vigorous stirring is performed mechanically This is continued for an hour or more since it is important to obtain a homogeneous suspension without clumps

A colloidal solution occurs during stirring and this cannot be separated by centrifugation The colloidal solution is brought into gel form by adding at the end of the stirring process a solution of 20 g sodium chloride (analytical product) in 500 ml distilled water Centrifugation is carried out immediately and the sediment is suspended by means of mechanical stirring in an amount of distilled water so that 4 kg gel is formed

During the above mentioned activation an adsorbent forms with affinities which are different from those possessed by the original Alhydrogel thus if a suspension of Y adsorbent is mixed with a suitable quantity of the original Alhydrogel spontanous flocculation of the two components occurs

The adsorbing capacity of Y adsorbent is controlled by measuring the ability to adsorb the protein from a one per cent solution of pure gamma G globulin in 0 9 per cent sodium chloride solution at pH 7 0 1 gr of the activated $Al(OH)_3$ in Y adsorbent should be able to adsorb nearly 1 gr of the globulin The adsorption is accomplished by a series of 5 times 10 cm³ Y adsorbent from a syringe which is fitted with a canula no 6 in the above mentioned globulin solution after each addition the adsorbate is separated by centrifugation

The globulin solution resting after adsorption should not contain more than 1 per mille protein when measured after the biuret technique

Before use the Y adsorbent must always be stirred to a homogeneous gel

Titration of the Consumption of the Adsorbent

During work where Alhydrogel and its derivatives are used for adsorption of proteins e g serum proteins flocculation titrations may be an aid in determining the consumption of the adsorbent Before carrying out the test we prepare two series each consisting of five tubes

Series I 10 ml of distilled water in each tube To each tube is added a dilution of the adsorbent

1 + 4 in distilled water, in increasing quantities e.g. 1 ml, 2 ml, 3 ml etc. The contents of the tubes are mixed thoroghly to obtain colloidal solutions of the adsorbent.

Series 2. Each tube contains 10 ml of a suitable dilution of protein solution in distilled water or saline, e.g. 1 + 4.

The contents of the tubes in Series 1 are mixed with the corresponding tubes in Series 2. After some minutes, flocculation can be observed in one of the tubes which soon develops into full sedimentation. This test provides a guide for calculation of the consumption of the adsorbent at each stage of the adsorption process.

Aerosil: Degussa, Weissfrauenstrasse 9, D 6 Frankfurt (Main) 1. This is a light powder consisting of pure silicium dioxide.

Freeze drying is conducted in an apparatus supplied by USIFROID, Procedés Rieutord, 9 rue Moreu Vauthier, Boulogne Seine.

Barium acetate standard solution: 5 kg barium acetate is dissolved in 9 litres distilled water, giving 14.5 litres of solution, to which is added 280 ml glacial acetic acid. pH in the reagent 5.5.

Acid Activation of Alhydrogel

Acid activation of Alhydrogel is carried out by use of polyphosphoric acid 84 per cent P_2O_5 (formula: $H\ P_4O_{13}$, chain length: 4 P atoms) from Knapsack Aktiengesellschaft, Griesheim.

A 10 per cent dilution of the acid in distilled water is prepared immediately before use because of the rapid hydrolysis of polyphosphoric acid in water systems.

1. *Acid Alhydrogel pH 2.5*: 3600 gr Alhydrogel is subjected to vigorous stirring, and at the same time about 30 ml of the 10 per cent dilution of the polyphosphoric acid is added by means of an injectionsyringe, which is fitted with canula no. 6. The time required for this addition is about 3 min. The pH must be checked. The adsorbent must be used immediately after preparation.

2. *Acid Alhydrogel pH 4.7*: In the same manner as mentioned above, 3400 g Alhydrogel is activated with about 6 ml of the 10 per cent dilution of polyphosphoric acid. The pH must be checked and the adsorbent must be used immediately after preparation.

Routine Assays Used

Tiselius electrophoresis is conducted in the Deptm. of Biophysics, Statens Seruminstitut, Head: A. Birch Andersen, M. Sc.

Immunoelectrophoresis is conducted in the Biochemical Department of Statens Seruminstitut. The gravimetric technique for estimation of blood proteins.

β lipoproteins are controlled by the technique described by *Heugel and Grunewald* (1967), under use of specific anti β lipoproteins prepared by immunization of rabbits with antigens after *Burstein and Samaille* (1958).

The *immuno-electrophoresis* has been performed on every step of the fractionation procedures by applying 5 ul antigen sample (total protein 50 g/l) in the central application hole on agar slides (*Clausen* 1960) (1 g DIFCO special Noble agar/100 ml 0.05 M Na barbital buffer pH 8.6). The electrophoresis was performed at 7 volt/cm for 1 hour and 15 minutes. The immunodiffusion (16 h) was performed after application of 40 ul horse anti human antiserum (Biotest Serum Institut, Frankfurt).

Description of the gravimetric technique for determination of proteins *Hinsberg and Lang* (1938).

Chemicals used: *acetate buffer* prepared from 56 ml glacial acetic acid, 1[illegible] g sodium acetate pro analysis, distilled water up to 1 litre.

(1) *Total protein*: 1 ml of plasma or serum, 3 ml of acetate buffer and 5 drops of saturated ammonium sulphate solution is put into a test tube which is placed in a boiling water bath for 30 minutes.

The coagulated protein is collected on a small filterpaper which is dried to a constant weight at 105°C.

The filterpaper is washed with distilled water until negative sulphate reaction is obtained. Thereafter is washed with a mixture of alcohol-ether (3:1) and at last with pure ether. The filter is dried to constant weight at 105° C.

(2) *Albumin fraction*: In a test tube is mixed 1 ml of plasma or serum, 2 ml of distilled water and 3 ml of saturated ammonium sulphate solution. After standing for at least 2 hours, the mixture is centrifugated for 10 minutes (at 6000 × g). From the clear supernatant, 3 ml is pipetted into a test tube and mixed with 3 ml of acetate buffer. The test tube is placed for 30 minutes in a boiling water bath, and then is filtered, washed, dried and weighed in the same manner as above.

What is weighed is the albumin in 0.5 ml serum.

Chemicals used are

L-glutamic acid: Riedel de Haen 27647
Methylenchloride: Riedel de Haen 24233
Bariumacetate pro analysis: Merck 1704
Polyphosphoric acid: Knapsack Griesheim, 84 per cent P_2O_5
Kal gnost: Heyl & Co. Hildesheim 1166

RESULTS

A description is given here of the production of Alb glut. from about 35 litres of bovine plasma, and from Alb glut. to pure crystalline bovine albumin.

The globulin was desalinated from 35 litres of bovine citrate plasma by semi saturation

with ammonium sulphate (2 1 M) at room temperature and neutral pH The clear supernatant fluid after centrifugation (6000 x g) containing among other proteins the plasma albumin was weighed and stirred mechanically About 12 g L—glutamic acid per litre was added and stirring was continued until the pH was 4 05 to 4 10 This should be achieved after 15 minutes The crude Alb glut was precipitated during stirring The mixture was left to stand overnight at 2°C

The product Alb glut as collected by centrifugation (6000 x g) has a satisfactorily constant of protein as estimated by repeated total protein determinations under use of the gravimetric technique described by *Hinsberg and Lang* (1938) For routine purposes it is important that the product is weighed since it is of interest to know this value when the subsequent calculations of yields are to be made The protein contained in the crude Alb glut is mainly albumin By repeated analysis by use of the gravimetric technique we could estimate about 85 per cent of the protein to be albumin As contaminating proteins were found under use of immunoelectrical technique to the homologous anti total immunsera that β globulins are present Besides the presence of several other plasma proteins could be observed The crude Alb glut was suspended in a mechanical mixer in a quantity of distilled water sufficient to make the volume 9 10 litres Adjustment was made to pH 5 5 by means of 2 n aquous ammonia solution during which a brown solution was formed Stirring was continued and during this the system was saturated with methylene chloride by addition of 2 vol per cent

A quantity of standard barium acetate solution corresponding v/w to the amount of the weighed Alb glut was added Centrifugation (6000 x g) was made the solid material was discarded

The centrifugate was liberated for its content of Ba^{++} by addition of ammoniumsulphate in a quantity calculated after titration of a sample under use of sodium rhodizonate as indicator *MAHR* (1961) The precipitated bariumsulphate was removed by centrifugation (6000 x g) and the centrifugate was acidified to pH 4 7 by addition of hydrochloric acid 2n α amylase solution was added in an amount corresponding to 10 ml per kg of the weighed Alb glut

The enzymic degradation took place during the night when the fluid was covered by a layer of toluene Temperature about 20°C No change in pH of the system was observed in this period The toluene was removed from the system by means of decanting The pH of the system was adjusted to 5 5 by means of 2n ammonia solution after which barium acetate was added in a quantity to secure a Ba^{++} concentration $\sim$ 0 02n in the final solution calculated after titration of a sample under use of sodium rhodizonate as indicator *MAHR* (1961)

Aerosil Adsorption for Removal of Transferrin

The conditions for adsorption of transferrin from the crude Alb glut (vide supra) by Aerosil are favourable at pH 5 5 where albumin has a negative charge and with an excess of $barium^{++}$ Aerosil (= pure silicium oxide) has a high affinity for transferrin while albumin remains refractory against this adsorbent in the same milieu

For adsorption a quantity of Aerosil No 200 was used corresponding to 5 per cent of the weighed Alb glut This light powder was poured on the surface of the fluid while it was stirred vigorously It could be seen when the mixture became homogeneous and centrifugation was carried out at once The system had lost its brown colour and immunelectrophoretical control showed that transferrin was eliminated

As contaminants were still to be found β lipoproteins by the technique of *Heugel & Gruneuald* (1967) and traces of β globulin and traces of γ globulin

Alhydrogel Adsorption for Removal of Lipoprotein and Traces of Globulins

During stirring a sufficient quantity of hydrochloric acid 2 n was added so that pH of the system became 4 2 At this pH β lipoprotein could be adsorbed selectively from

the albumin, under use of Alhydrogel acid-activated to pH 2 5 by means of polyphosphoric acid – The adsorption conditions could be controlled by flocculation titration which makes it possible to find the limit for adsorption of β lipoprotein The supernatant after the adsorption shows not to contain β lipoprotein controlled after *Heugel & Grunewald* (1967)

As contaminant was found traces of β and γ-globulines by immunophoretical control These globulines could be adsorbed from the system under use of Alhydrogel acid activated to pH 4 7 with polyphosphoric acid

The quantity of adsorbent was determined by flocculation titration However, since it has been found that consumption of Alhydrogel is in a satisfactorily constant relation to the weighed amount of Alb glut, a technique can be adopted without prior examination If the amount of Alb glut is 4000 g the following applies

1st adsorption stage 3600 g Alhydrogel activated at pH 2 5

2nd adsorption stage 3100 g Alhydrogel activated at pH 4 7

Adsorption takes place when the activated Alhydrogel is poured slowly into the system while it is being stirred Centrifugation can be carried out immediately after adsorption

After the double adsorption with Alhydrogel is completed the fluid is a clear and colourless solution of albumin pH 4 2 The pH is raised to 5 5 by means of 2 n ammonia solution after which the system is made semi saturated with ammonium sulphate 290 g of the salt per litre being dissolved After being allowed to stand for some hours the fluid is filtered in order to remove the precipitated $BaSO_4$

The Alb glut is crystallized by acidification to pH 4 05 by the addition of 2 n hydrochloric acid during stirring During the crystallization a consumption of acid occurs It has been found by measuring after 30 minutes that the pH has risen to about 4 30 Repeated acidification to pH 4 05 is thus important for achieving total crystallization

After standing overnight the crystallized product is collected by centrifugation It is then dissolved in distilled water simultaneously with the addition of 2 n sodium hydroxide solution so that the solution has a pH of 7 0

In addition to albumin, the solution contains the salts sodium glutamate and ammonium sulphate In order to remove these salts the solution is dialysed against distilled water (abt 75 litres), which is stirred and changed at regular intervals The dialysis time can be shortened considerably if the dialysis water is heated to 50 55°C

After conclusion of dialysis, the solution is filtered through Seitz EK plates and the albumin is dried by lyophilization The yield from about 35 litres of bovine plasma is about 200-250 gr of pure crystalline albumin controlled by immunoelectrophoresis for protein contaminant and absence of β lipoproteins after *Heugel & Grunewald* (1967)

Using the technique described albumin of immunoelectrophoretically (vide infra) pure quality can be produced from all crude substances that contain albumin and therefore from plasma and serum containing haemoglobin Special mention should be made of placental blood which is extracted by centrifugation of chopped placenta Placental blood is particularly strongly contaminated with haemoglobin, cholesterol and lipoprotein and has a relatively high content of hormones and enzymes

Since cord blood is readily available crude material for the production of pure crystalline human albumin it is considered of value to mention here the special pre treatment recommended in order to utilize placental blood in the same way as plasma or serum

Pre treatment of Placental Blood

The placental blood is obtained from disintegrated placenta by centrifugation in a basket centrifuge (1200 x g) From 100 placenta a yield of about 20 liter blood could be achieved

Another important source for raw material is cord blood which is also collected in the clinics during the birth As raw material for

with ammonium sulphate (2 1 M) at room temperature and neutral pH The clear supernatant fluid after centrifugation (6000 x g) containing among other proteins the plasma albumin was weighed and stirred mechanically About 12 g L—glutamic acid per litre was added and stirring was continued until the pH was 4 05 to 4 10 This should be achieved after 15 minutes The crude Alb glut was precipitated during stirring The mixture was left to stand overnight at 2°C

The product Alb glut as collected by centrifugation (6000 x g), has a satisfactorily constant of protein, as estimated by repeated total protein determinations, under use of the gravimetric technique described by *Hinsberg and Lang* (1938) For routine purposes, it is important that the product is weighed, since it is of interest to know this value when the subsequent calculations of yields are to be made The protein contained in the crude Alb glut is mainly albumin By repeated analysis, by use of the gravimetric technique, we could estimate about 85 per cent of the protein to be albumin As contaminating proteins were found, under use of immunoelectrical technique to the homologous anti total immunsera, that β globulins are present Besides the presence of several other plasma proteins could be observed The crude Alb glut was suspended in a mechanical mixer in a quantity of distilled water sufficient to make the volume 9-10 litres Adjustment was made to pH 5 5 by means of 2 n aquous ammonia solution, during which a brown solution was formed Stirring was continued, and during this, the system was saturated with methylene chloride by addition of 2 vol per cent

A quantity of standard barium acetate solution, corresponding v/w to the amount of the weighed Alb glut, was added Centrifugation (6000 x g) was made, the solid material was discarded

The centrifugate was liberated for its content of Ba^{++} by addition of ammoniumsul [illegible] after titration [illegible] as indicator *MAHR* [illegible] bariumsulphate was removed by centrifugation (6000 x g), and the centrifugate was acidified to pH 4 7, by addition of hydrochloric acid 2n α amylase solution was added in an amount corresponding to 10 ml per kg of the weighed Alb glut

The enzymic degradation took place during the night, when the fluid was covered by a layer of toluene Temperature about 20°C No change in pH of the system was observed in this period The toluene was removed from the system by means of decanting The pH of the system was adjusted to 5 5 by means of 2n ammonia solution, after which barium acetate was added in a quantity to secure a Ba^{++} concentration $\sim$ 0 02n in the final solution calculated after titration of a sample, under use of sodium rhodizonate as indicator *MAHR* (1961)

Aerosil Adsorption for Removal of Transferrin

The conditions for adsorption of transferrin from the crude Alb glut (vide supra) by [illegible] has a high affinity for transferrin, while albumin remains refractory against this adsorbent in the same milieu

For adsorption, a quantity of Aerosil No 200 was used corresponding to 5 per cent of the weighed Alb glut This light powder was poured on the surface of the fluid while it was stirred vigorously It could be seen when the mixture became homogeneous and centrifugation was carried out at once The system had lost its brown colour, and immunelectrophoretical control showed that transferrin was eliminated

As contaminants were still to be found β lipoproteins by the technique of *Heugel & Grunewald* (1967) and traces of β_1-globulin and traces of γ-globulin

Alhydrogel Adsorption for Removal of Lipoprotein and Traces of Globulins

During stirring, a sufficient quantity of hydrochloric acid [illegible] n was added so that pH of the system became 4 2 At this pH β lipoprotein could be adsorbed selectively from

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THE FATE OF HETEROLOGOUS SPECIFIC AND NON-SPECIFIC ANTILYMPHOCYTE GLOBULINS IN PLASMA AND TISSUES STUDIED BY A PAIRED-LABEL TECHNIQUE

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The elimination and tissue distribution of different batches of rabbit antimurine antilymphocyte globulin and antihuman antilymphocyte globulin were studied by means of a paired label technique after injection into mice The identical over all clearance rates of antimurine ALG and control rabbit IgG judged by whole body count compared with a slightly lower plasma level of ALG indicated an increased binding of the injected ALG to tissues The plasma deficit of ALG was balanced by a higher uptake in the spleen liver and kidney but not in the thymus In particular the spleen was found to exhibit a much higher avidity for ALG than for normal IgG while the actual elimination rates for the two globulins were the same for spleen other tissues and plasma The interpretation follows that no accumulation of "fixed ALG occurred within lymphoid organs which would have been expected if the "blindfolding" theory of the immunosuppressive effect of ALG is correct This difference in avidity was roughly correlated to the specificity of the ALG preparation used

The introduction of heterologous antilymphocyte globulin (ALG) as a potent immunosuppressive agent in tissue transplantation raised the problem of the rate limiting effect of ALG on immunosuppression due to the immunogenic nature of the foreign protein The effect of the immunogenicity of ALG on graft survival antibody response, tolerance induction and maintenance has been investigated by several workers (*James & Anderson* 1967 *Ono et al* 1969 *Wood* 1970) In turn-over studies employing radiolabelled ALG and/or IgG the former appeared to be more immunogenic than normal heterologous IgG when tested after repeated injections of unlabelled immunoglobulins (*Clark et al* 1967, *Lance & Dresser* 1967, *Jasin et al* 1968) While plasma elimination studies roughly reflect the net balance of ALG and IgG in the whole animal more information regarding the mode of action of ALG may be obtained by studying the fate of injected ALG and IgG in individual organs

In the present study we have found a significant difference between the distribution of ALG and IgG in the lymphoid organs of normal mice as compared to other tissues and blood This difference can be roughly

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correlated to *the degree of specificity of the* ALG used Our results do not favour the hypothesis of lymphocyte "blindfolding" by ALG, originally suggested by *Levey & Medawar* (1966)

MATERIAL AND METHODS

Animals Female inbred mice of the C3H strain, weighing 21–25 g, were used throughout One week prior to and during the experiments 0 2 per *cent potassium iodide was added to the drinking* water to block thyroid uptake of radioiodine The animals were kept in separate cages provided with steel mesh floors to avoid major isotope contamination of the fur

Production of ALG Rabbit antimouse antithymus sera were prepared in albino rabbits according to the two pulse i v method of *Levey & Medawar* (1966) The pooled sera were *inactivated* at 56° C for 30 min Sera from normal rabbits were treated similarly Antilymphocyte globulin (ALG) and normal rabbit IgG were prepared from antilymphocyte serum and normal rabbit serum by standard methods employing DEAE column chromatography The purity of the final preparations was verified by immunoelectrophoresis *In vivo* properties were judged by the ability of the ALG *to prolong skin allografts in C3H mice* Rabbit antihuman ALG was prepared in the same manner, using human peripheral lymphocytes as antigen (The antimouse ALG employed in the experiment described under Protocol 1 was produced in collaboration with Drs Nils Axelsen and Niels Harboe, The Protein Laboratory, University of Copenhagen The antihuman ALG was kindly supplied by J V Sparck Ph D State Serum Institute Copenhagen)

Radiolabelling of ALG and IgG A paired label technique was used in order that each mouse should serve as its own control Purified ALG in an amount of 25 mg was labelled with ^{131}I while the same amount of normal rabbit IgG (in the following referred to as IgG) was labelled with ^{125}I The labelling was carried out by the iodine monochloride method of *McFarlane's* (1958) The proteins were labelled with less than two iodine atoms per molecule The specific activity was 22 μCi/mg To the labelled preparation was added 1 0 ml of unlabelled IgG in a 1 per cent solution as carrier bringing the final volume of the preparation to 5 6 ml Less than 0 5 per cent of the iodide was non protein bound

Blood sampling and tissue preparations At various intervals 25 μl of blood was drawn from the retroorbital plexus and diluted with 3 ml distilled water The animals were killed and bled out by decapitation The spleen liver, kidney, lung and thymus were removed After the determination of the wet weight of each organ these were dissolved in 3 ml of 30 per cent potassium hydroxide at 90° C

Measurement of radioactivity Whole body activity was measured on anaesthetized animals observing standardized geometries in a 7×7×10 cm chamber shielded with two inches of lead The bottom of the chamber was a gamma detector with a 3 inch NaI (Tl) crystal connected to a pulse height analyzer ^{131}I and ^{125}I activities were counted separately ^{131}I and ^{125}I activities of blood samples and tissue hydrolysates were counted simultaneously using a well type NaI(Tl) crystal connected with a dual channel gamma spectrometer (Packard 3002) A standard preparation of the injected proteins was counted at the same time

Calculations The intravascular activity was calculated by assuming that 10 minutes after intravenous injection 98 per cent of the tracer was still in the plasma while 2 per cent had passed to *the extravascular compartments Furthermore, it* was assumed that the plasma volume constituted 5 per cent of the weight of the animal and that the hematocrit value was 0 40 In whole organs, the *relative* distribution of ALG and IgG as a function of time was calculated by assuming a ratio ALG IgG in the 10 min plasma sample of unity The specific activities of ALG and IgG per mg tissue were calculated from the weight of the counted organs These were compared with the corresponding activities per volume unit plasma

Protocol 1 10 mice were injected by the intravenous route with a mixture of 0 8 mg rabbit ^{131}I ALG and 0 8 mg rabbit ^{125}I IgG in 0 3 ml saline The injected activities represented approximately 5 μCi of ^{131}I and 12 μCi of ^{125}I Blood was drawn at the following intervals 10, 20 40, 60 and 120 *minutes after injection* and subsequently at days 1, 2 3 4, 5 6, 8 9, 11 and 14 From day 2 and onwards whole body countings were carried out at regular intervals until day 13 Fourteen days after injection the animals were killed and the tissues were prepared as described above

Protocol 2 20 mice were injected intravenously [illegible] ...shed [illegible]

from each animal The mice were killed off in groups of five after 3 hours, 1 day, 2 days and 7 days respectively Blood and tissues were removed and prepared as described above In this experiment a batch of ALG which was slightly less immuno- [illegible] ...sed [illegible] ...enously [illegible] ...I and [illegible] ...I The experimental procedures were otherwise identical to those described in protocol 2

RESULTS

The results from the experim... 1 are depicted in Figs 1–4 and T... 1 and 2 are both drawn on a se... It is immediately apparent from Fig... initially ALG disappears slightly ... the blood stream than IgG This is ... in Fig 2 which also shows that from ... the plasma elimination curves of b... teins are monoexponential with ... rate constants (parallel in semilog scale... From the start however a slightly sma... fraction of the injected ALG than of the IgG persists in the plasma The slight d... at day 15 signifies the start of the anticipated immune (non metabolic) elimination of the foreign globulin The graph at the top of Fig 2 showing the whole body counts exhibit— with due approximation—merging curves for ALG and IgG These curves run parallel (i e in a semilog scale) with those represent

THYMUS

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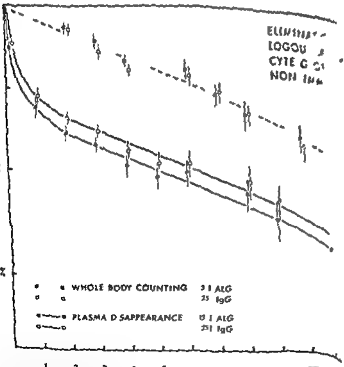

Fig 2 Whole body Counts and Plasma Elimination of ^{131}I ALG and ... After Intravenous Injection Results Expressed as Mean Values for ... Mean (S E M)

7 DAYS

iues) Calculated
G and ^{125}I IgG
Mice Killed at

ing plasma elimination (same half life) The differing concentrations of ALG and IgG in the plasma are neatly balanced by a comparatively higher ALG content within certain tissues as shown in Table 1 14 days after injection the amount of ^{131}I-ALG and ^{125}I IgG persisting expressed as a percentage of the radioactivity injected at day 0, is almost identical (10 21 per cent and 10 85 per cent) when calculated for tissue and plasma together, *corresponding to the pattern of identity* shown by whole body elimination curves However, single organs showed quite a different pattern which can be more clearly seen in Fig 3 Calculations of the ratio ALG IgG demonstrated a higher avidity of the tissues for ALG—particularly in the spleens but also in the kidneys and livers No preferential uptake of ALG was noted in the thymus Fig 4 shows that the spleen and kidney contain more ALG than the other organs when differences in weights are disregarded Thus the spleens contained 70 per cent of the ALG and 8 per cent of the IgG *present in plasma The corresponding* values for the thymus were 11 per cent and 9 per cent respectively

TABLE 1 *Distribution in Whole Organs of Anti mouse ALG and Control IgG (Normal Rabbit IgG) in Mice 14 Days after Intravenous Injection*

	IgG	ALG
Blood	9 50 %	7 60 %
Spleen	0 05 %	0 22 %
Liver	0 75 %	1 49 %
Kidney	0 24 %	0 52 %
Lung	0 28 %	0 34 %
Thymus	0 03 %	0 04 %

Protocol 2 was designed to elucidate whether the above mentioned differences in organ binding of ALG and IgG represented a steady or a dynamic state The results are illustrated in Figs 5 and 6 In Fig 5 mean values for the amount of ^{131}I ALG and ^{125}I IgG persisting in plasma and various tissues are plotted for groups of mice killed after varying time intervals following the injection The top left hand graph—representing plasma—is comparable to that of Fig 2 showing

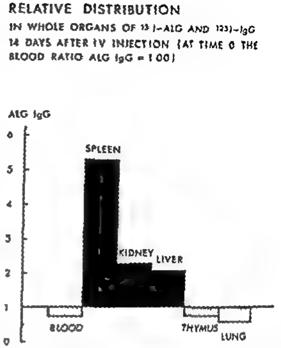

Fig 3 ALG Content in Various Tissues Related to the Content of IgG Mean Values from 10 Mice 14 Days after Injection

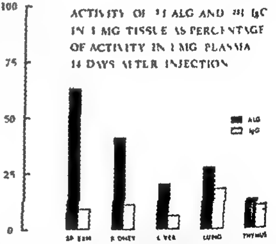

Fig 4 Activity of ^{131}I ALG and ^{125}I IgG as Percentage of Activity in 1 Mg Plasma 14 Days After I v Injection

that both the injected globulins have the same half life Both ALG and IgG are eliminated from the lungs at the same rate as from plasma and at any given time interval are present in the same quantities In contrast liver kidney and in particular the spleen exhibit a much higher avidity for

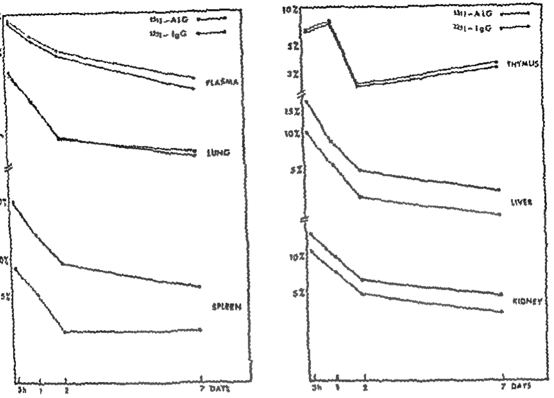

Fig 5 Mean Values for Residual Activities of 131I-ALG and 125I-IgG in Plasma and Various Tissues Plotted for Groups of Mice Killed After Varying Periods Following Injection Semilog Scale

ALG than for IgG The rates of elimination of ALG and IgG from these tissues are the same as the rates of clearance from plasma and lung, a half-life of approximately 7 days In other words, the IgG content of the organs was lower than that of ALG at any given moment, in spite of identical elimination rates This is seen as the spatial difference between the elimination curves for the various tissues examined, which is most pronounced in the spleen In this respect the graph depicting elimination from the thymus differs completely The uptake of ALG and IgG is slower, the two labelled proteins are eliminated in a peculiar fashion, but at the same rate and with equal amounts present throughout the experiment

Fig 6 illustrates the same findings expressed as ALG IgG ratios in plasma and whole organs The configuration of each of the columns in the diagram closely resembles that of Fig 3 which was obtained 14 days

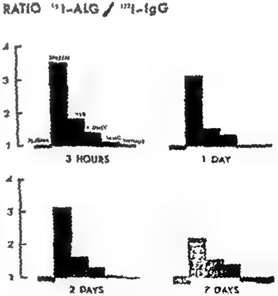

Fig 6 ALG IgG Ratios (Mean Values) Calculated From Residual Activities of 131I ALG and 125I IgG in Plasma and Various Tissues of Mice Killed at Varying Intervals After Injection

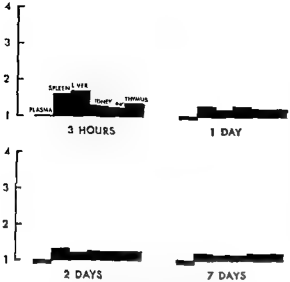

Fig 7 ALG IgG Ratios (Mean Values) Calculated From Residual Activities of ^{131}I ALG and ^{125}I IgG in Plasma and Various Tissues in Groups of Mice Killed After Various Intervals Following the Injection at Day 0 of a Mixture of 0 8 Mg Rabbit Antihuman ^{131}I ALG + 0 8 Mg Normal Rabbit ^{125}I IgG Intravenously

after the injection of a different batch of ALG

Protocol 3 aimed at the influence of specificity on the distribution and elimination of heterologous ALG and IgG It can be seen from Fig 7 that after 3 hours a slight and probably significant excess of antihuman ALG was evident in murine spleens and livers in comparison with the distribution of normal IgG Hereafter, the counts were within the limits set by inherent experimental errors

DISCUSSION

From the results depicted in Figs 1, 2 and 5 it may be concluded that—under our experimental conditions—both normal rabbit IgG and rabbit antimouse ALG had approximately the same half-life in plasma after a single intravenous injection in normal mice "Immune elimination" was not observed until 15 days after injection Whole body counts revealed indentical patterns for the elimination of both ^{131}I-ALG and ^{125}I-IgG, while the plasma counts exhibited a minor vertical displacement of the elimination curve for ALG, due to an initially steeper slope Earlier studies by *Lance* & *Dresser* (1967) in which mice were injected intraperitoneally with radiolabelled normal rabbit IgG indicated a mean half-life in the plasma of about 5-6 days for this globulin, which corresponds fairly well with our findings However, Lance & Dresser found an increased elimination rate for rabbit antimouse ALG resembling that of a secondary immune response, which has not been confirmed in the present experiments *Jann et al* (1968) also found ALG to be more immunogenic in rats than normal IgG, but these authors administered the globulins as three consecutive injections over 3-day periods Paired label techniques were not employed Because of this fact and because both these groups injected ALG and IgG by the intraperitoneal route their results cannot be directly compared with ours, and therefore the observed discrepancy may be an apparent one

In the present experiment the identical over-all clearance rates of ALG and IgG judged by whole-body counts compared with the slightly lower plasma level of ALG indicated an increased binding of the injected ALG to tissues From Table 1 and Figs 3-4 it can be seen that the plasma deficit of ^{131}I ALG is balanced by a higher activity of ^{131}I in spleen, liver and kidney The accumulation of ALG in the spleen is striking especially when compared with the identical distribution of ALG and IgG in the thymus It should be remembered that the antigen employed in the production of ALG was murine thymocytes The repeated findings in our experiments of a high splenic uptake of ALG are not in accordance with the results obtained by some other investigators (*Hintz* & *Webber* 1965, *Denman* & *Frenkel* 1968 *Nava et al* 1969) but are similar to results reported by *Guttman et al* (1967 Our findings of a limited thymus uptake of ALG however, is only unequivocally confirmed in the available literature, some reports are in accordance (*Denman* & *Frenkel* 1968 *Lance*

1969), others are not (*Hintz & Webber* 1965 *Guttman et al* 1967, *Naza et al* 1969) An analysis of these discrepancies is not facilitated by the wide range of experimental designs employed by the various investigators

Two alternative explanations for the high spleen ratio (4 1) of ALG to IgG two weeks after a single injection of a mixture of the two globulins were considered 1) An early binding of a fixed amount of ALG to the lymphoid organ would result in a stationary fraction of (monoclonal) ALG which in time would increase in relation to the decreasing amounts of ALG linked globulins in plasma and other tissues, 2) an initially higher inflow rate of ALG into the spleen rapidly counterbalanced by a proportional outflow Hereafter, the fractional inflow of ALG would remain higher than that of IgG Virtual fixation would be absent

The results shown in Figs 5 and 6 suggest that the second alternative is a plausible explanation The spleen exhibits a much higher avidity for ALG than for IgG, while the respective elimination rates from the spleen are the same as in plasma and other tissues Thus, it seems probable that the uneven organ distribution is not caused by a fixation of ALG in the lymphoid organs which exceeds that of normal IgG If this were so the elimination curves for ALG and IgG would diverge, which they do not (as seen for the spleen) The interpretation follows that no accumulation of fixed ALG occurred which would be expected if a blindfolding (*i e* coating" by ALG of *lymphoid cells*) theory on the mode of action of ALG was correct Even though one takes into consideration the role played by the recirculation of small lymphocytes within the lymphoid pool it seems unlikely that the rates of clearance of ALG and IgG from a lymphoid organ would be parallel if a fixed coating (blindfolding) of small lymphocytes by ALG occurred

The absence of specific ALG uptake (preferential binding) in the thymus and the peculiar pattern of elimination of both ALG and IgG from this organ fit accepted views on the restricted access of antibody to this organ (the 'blood thymus barrier')

Judged by graft survival the batch of ALG used in Protocol 1 was slightly better than the batch employed in Protocol 2 Accordingly, spleen uptake in the latter was found to be somewhat less pronounced This may feature a rough parameter of specificity, a view which is supported to some extent by the experiment with antihuman ALG (Protocol 3) the slight and temporary rise in the ratio ALG IgG in the mouse spleen shortly after injection (Fig 7) is compatible with a low degree immune cross reactivity between different mammalian species

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TWO HAEMAGGLUTINATING COMPONENTS OF VESICULAR STOMATITIS VIRUS

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Haemagglutinin of vesicular stomatitis virus, Indiana serotype, was propagated in BHK21/13S cells, using diluted seed virus and multicyclic growth. In a 15 to 45 per cent sucrose gradient two haemagglutinins with different sedimenting properties could be separated. The proportions of these haemagglutinins depended on the input multiplicity of infection. In preparations obtained with undiluted seed virus, the proportions of the more slowly sedimenting haemagglutinin was strongly increased. The infectivity was associated with the lower band. In electron microscopy virions were found in the lower band whereas the upper band consisted of so-called T particles and some rosettes. The two haemagglutinating components could also be separated by their densities in a CsCl gradient. The densities were 1.22 g/cm³ for the virion-containing band and 1.18 g/cm³ for the upper band. The two haemagglutinating components had the same protein composition in acrylamide gel electrophoresis. The results obtained suggest that, in addition to the virion, the shorter T particles also possess haemagglutinating capacity.

Vesicular stomatitis virus (VSV) has earlier been reported to possess haemagglutinating activity (1,7). The optimal conditions for VSV haemagglutination (HA) included the use of goose erythrocytes, low temperature and pH 5.8. The haemagglutinating activity seemed to be associated with the virion only.

Both serotypes of VSV, Indiana and New Jersey, exhibit the phenomenon of auto-interference (4, 6, 8, 12). If host cells are infected with a high multiplicity of infection (m.o.i.) they produce a low yield of infective virus but a high yield of so-called T particles (truncated or transmissible). This particle is only about one-third of the length of the virion and is therefore also called "S" for short (5). Whole bullet-shaped virions are called "B" for bullet or "L" for long, respectively.

The present report demonstrates that there is another VSV fraction, in addition to the virion, that possesses haemagglutinating activity. This fraction is associated with the T particles.

MATERIALS AND METHODS

Preparation of haemagglutinin. Throughout the whole study the same seed virus, prepared from the prototype strain of VSV Indiana serotype, was used. Previously it had been passed twice in BHK 21/13 S cells in this laboratory. Indiana serotype was mainly used, but some experiments were also performed with New Jersey serotype, provided by the American Type Culture Collection. The infectivity titre of the seed virus, Indiana serotype, was 4×10^9 plaque forming units (PFU)/ml. Haemagglutinin was produced as described earlier (1),

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using a multicyclic growth in a suspension culture of BHK 21/13 S cells maintained in BHK 21 medium with 0.4 per cent bovine albumin, 10 per cent tryptose phosphate broth and no serum. Multiplicities of infection were 0.001 and 10 PFU/cell.

Purification and concentration. After removal of cells by low speed centrifugation, the extracellular virus was precipitated and partly purified by polyethylene glycol (PEG) 6000 precipitation (11). To 100 ml of virus 2.2 g of NaCl and 6 g of PEG was added. Precipitation was allowed to take place overnight at 4° C with constant stirring. The precipitate was pelleted by centrifuging at 1000 g for 15 minutes and eluted in 10 ml of 0.05 M tris (hydroxymethyl) aminomethane buffer, pH 7.4, containing 1 mM of EDTA and 0.15 M NaCl. The virus was pelleted again at 20,000 r.p.m. for 90 minutes in the 30 rotor of a Model L-50 Spinco ultracentrifuge and the pellet resuspended in 1 ml of the same buffer. Finally, this resuspended virus was layered on top of 29 ml of 15 to 45 per cent sucrose gradient and centrifuged for 2 hours at 22,500 r.p.m. in the Spinco SW 25 rotor. Fractions of 20 drops, approximately 1,5 ml each, were collected. These fractions were assayed for complement fixation (CF), HA and infectivity. Fractions suitable for electron microscopy and for polyacrylamide gel electrophoresis were pooled and the sucrose gradient centrifugation was repeated. The pooled fractions were dialysed against 0.05 M tris-EDTA-NaCl or 0.01 M phosphate buffer, pH 7.0, to remove sucrose.

Serological methods. Haemagglutination tests were performed as described earlier (1) by a microtitre technique, with 0.4 per cent bovine albumin borate saline as diluent, pH 5.8, goose erythrocytes and ice water temperature. The volume of haemagglutinin dilutions used for titrations was 0.05 ml. The highest dilution causing complete agglutination was taken as the haemagglutinin titre.

In complement fixation test antigens were titrated with 4 units of anti-VSV rabbit hyperimmune serum (1) and 2 units of complement. The amount of antibody was standardized by box titration with the seed virus preparation of VSV described above. The volume of antigen dilutions was 0.025 ml. The 50 per cent haemolysis end point was estimated visually and taken as the CF antigen titre.

Plaque titrations. Infective virus was estimated by plaque titration in BHK 21/13 S agarose suspension cultures (13). One ml of BME diploid medium containing 5 per cent calf serum and 0.5 per cent agarose was allowed to harden as an underlayer in a 30 mm plastic Falcon Petri dish and 0.05 ml of the diluted virus suspension was added to 0.45 ml of cell suspension (10×10^6 cells/ml) in BHK 21 medium containing 2 per cent calf serum. The cell-virus mixture was incubated for 1 hour at 35° C to allow adsorption and penetration of the virus particles into the cells, and an equal amount of 0.5 per cent agarose in BME medium with 5 per cent calf serum was added at 44° C. A 0.25 ml portion of this mixture was pipetted onto the agarose underlayer of each Petri dish and spread evenly by rotating the dish. After incubation for 36 hours at 37° C in an atmosphere of 5 per cent CO_2 in humified air, the plaques were made visible by 0.75 ml of the same 0.5 per cent agarose in BME now containing 10^{-4} g of neutral red per ml. The plaques, 3 to 4 mm in diameter, were counted some hours later.

Electron microscopy. A standard negative staining method was used. A drop of each sample was placed on a formvar-covered grid and the excess was drained with a filter paper. The specimen was allowed to dry and was stained with 2 per cent phosphotungstic acid (PTA) pH 7.0 for 30 seconds. The specimens were examined in a Siemens Elmiskop I A at an instrumental magnification of 30 000.

Polyacrylamide gel electrophoresis. The pooled sucrose fractions containing purified antigens were first dialysed overnight at 4° C against 100 volumes of 0.01 M phosphate buffer pH 7.0, to remove the sucrose. The buffer was changed once. The protein concentration of each sample was determined according to Lowry's method and measured in a Hitachi Perkin Elmer 139 Spectrophotometer. The samples were diluted to contain the same concentration of protein, if necessary.

Sodium dodecyl sulphate (SDS) and 2-mercapto-ethanol (2 ME) were added to the samples, to a final concentration of 1 per cent for each, and the samples were heated to the boiling point. After this treatment the mixture was dialysed overnight at room temperature against 100 volumes of 0.01 M phosphate buffer containing 0.1 per cent SDS and 0.1 per cent 2 ME. Acrylamide gel columns were prepared and running of electrophoresis was performed according to Maizel (10). The samples were mixed with 60 per cent sucrose 4:1 and 0.3 ml of each was layered on polymerized acrylamide gel columns. The acrylamide gel contained 5 per cent acrylamide and 0.1 per cent SDS. The running buffer 0.1 M sodium phosphate buffer was made in 0.1 per cent in SDS. The lengths of the columns varied in the different tests and accordingly the times of run, but a voltage of 50 V and current of 25 to 50 mA was used in each test. After 2 to 8 hours the protein bands were fixed overnight with 20 per cent sulphosalicylic acid and stained with 0.25 per cent Coomassi blue for 6 hours. The excess of stain was washed with 7 per cent acetic acid.

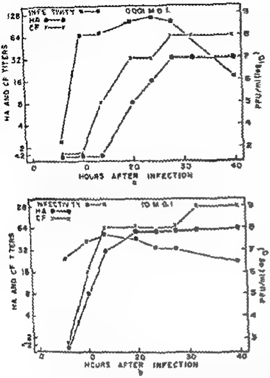


Fig 1 Development of haemagglutinating, complement fixing and infective activities of vesicular stomatitis virus grown in BHK 21/13 S cells with input m o i of 0.001 (a) and 10 (b) plaque forming units per cell

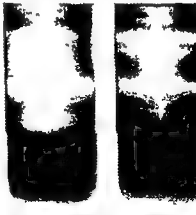
RESULTS

Development of Haemagglutinating Complement Fixing and Infective Activities

When the usual multiplicity of infection 0.001 PFU/cell was used extracellular haemagglutinin could not be detected until 16 to 20 hours after infection (Fig 1a). Complement fixing antigens were present in the extracellular liquid 3–6 hours earlier than haemagglutinin Haemagglutination titres reached the peak level in 28 to 36 hours and then remained stable for a further 24 hours The infectivity reached the peak in 20 to 24 hours and began to decrease 28 hours after infection The CF activity increased steadily during the first 28 hours and then remained stable If 10^4 times greater multiplicity of infection was used (Fig 1b) detectable haemagglutinin was produced as early as 10 hours after infection and the peak level was usually reached in 16 to 20 hours Infective viruses were also formed earlier than otherwise seen, but the titres remained at least 1 $\log_{10}$ lower

Sucrose Gradient Centrifugation of Haemagglutinin

For purification haemagglutinin was precipitated with PEG, eluted, and pelleted again by centrifuging at 20 000 r p m for 90 minutes in the Spinco 30 rotor About 20 per cent of the total haemagglutinin was constantly left in the supernatant The pelleted haemagglutinin was then layered on top of a 15 to 45 per cent linear sucrose gradient After centrifugation for 120 minutes at 22,500 r p m, two visible bands were formed (Fig 2a), the lower of which was the more prominent Fractions of 20 drops were assayed for HA and CF activities Two main peaks were obtained in both tests. The fractions containing the peak activities of HA and CF were associated with the bands visible in the ultracentrifuge tube The highest infectivity was found in the lower band and was thus associated with the more



a b

Fig 2 Light-scattering bands of concentrated vesicular stomatitis virus propagated with m o i of 0.001 (a) and 10 (b) plaque forming units per cell after centrifugation on 15 to 45 per cent sucrose gradient for 2 hr at 22 500 r p m in the Spinco SW 25 rotor

using a multicyclic growth in a suspension culture of BHK 21/13 S cells maintained in BHK 21 medium with 0 4 per cent bovine albumin, 10 per cent tryptose phosphate broth and no serum Multiplicities of infection were 0 001 and 10 PFU/cell

Purification and concentration After removal of cells by low speed centrifugation, the extracellular virus was precipitated and partly purified by polyethylene glycol (PEG) 6000 precipitation (11) To 100 ml of virus 2 2 g of NaCl and 6 g of PEG was added Precipitation was allowed to take place overnight at 4° C with constant stirring The precipitate was pelleted by centrifuging at 1000 [illegible] for 15 minutes and eluted in 10 ml of 0 05 M tris (hydroxymethyl) aminomethane buffer, pH 7 4, containing 1 mM of EDTA and 0 15 M NaCl The virus was pelleted again at 20,000 r p m for 90 minutes in the 30 rotor of a Model L 50 Spinco ultracentrifuge and the pellet resuspended in 1 ml of the same buffer Finally, this resuspended virus was layered on top of 29 ml of 15 to 45 per cent sucrose gradient and centrifuged for 2 hours at 22,500 r p m in the Spinco SW 25 rotor Fractions of 20 drops, approximately 1,5 ml each, were collected These fractions were assayed for complement fixation (CF), HA and infectivity Fractions suitable for electron microscopy and for polyacrylamide gel electrophoresis were pooled and the sucrose gradient centrifugation was repeated The pooled fractions were dialysed against 0 05 M tris EDTA NaCl or 0 01 M phosphate buffer, pH 7 0 to remove sucrose

Serological methods Haemagglutination tests were performed as described earlier (1) by a microtitre technique, with 0 4 per cent bovine albumin borate saline as diluent, pH 5 8, goose erythrocytes and ice water temperature The volume of haemagglutinin dilutions used for titrations was 0 05 ml The highest dilution causing complete agglutination was taken as the haemagglutinin titre

In complement fixation test antigens were titrated with 4 units of anti VSV rabbit hyperimmune serum (1) and 2 units of complement The amount of antibody was standardized by box titration with the seed virus preparation of VSV described above The volume of antigen dilutions was 0 025 ml The 50 per cent haemolysis end point was estimated visually and taken as the CF antigen titre

Plaque titrations Infective virus was estimated by plaque titration in BHK 21/13 S-agarose suspension cultures (13) One ml of BME diploid medium containing 5 per cent calf serum and 0 5 per cent agarose was allowed to harden as an underlayer in a 30 mm plastic Falcon Petri dish and 0 05 ml of the diluted virus suspension was added to 0 45 ml of cell suspension (10×10^6 cells/ml) in BHK 21 medium containing [illegible] per cent calf serum The cell virus mixture was incubated for 1 hour at 35° C to allow adsorption and penetration of the virus particles into the cells, and an equal amount of 0 5 per cent agarose in BME medium with 5 per cent calf serum was added at 44° C. A 0 25 ml portion of this mixture was pipetted onto the agarose underlayer of each Petri dish and spread evenly by rotating the dish After incubation for 36 hours at 37° C in an atmosphere of 5 per cent CO_2 in humified air, the plaques were made visible by 0 75 ml of the same 0 5 per cent agarose in BME now containing 10^{-4} g of neutral red per ml The plaques, [illegible] to 4 mm in diameter, were counted some hours later

Electron microscopy A standard negative staining method was used A drop of each sample was placed on a formvar covered grid and the excess was drained with a filter paper The specimen was allowed to dry and was stained with 2 per cent phosphotungstic acid (PTA) pH 7 0 for 30 seconds The specimens were examined in a Siemens Elmiskop I A at an instrumental magnification of 30,000

Polyacrylamide gel electrophoresis The pooled sucrose fractions containing purified antigens were first dialysed overnight at 4° C against 100 volumes of 0 01 M phosphate buffer, pH 7 0, [illegible] remove the sucrose The buffer was changed once The protein concentration of each sample was determined according to Lowry's method and measured in a Hitachi Perkin Elmer 139 Spectrophotometer The samples were diluted to contain the same concentration of protein, if necessary

Sodium dodecyl sulphate (SDS) and 2 mercapto ethanol (2 ME) were added to the samples, to a final concentration of 1 per cent for each, and the samples were heated to the boiling point After this treatment the mixture was dialysed overnight at room temperature against 100 volumes of 0 01 M phosphate buffer containing 0 1 per cent SDS and 0 1 per cent 2 ME Acrylamide gel columns were prepared and running of electrophoresis was performed according to Maizel (10) The samples were mixed with 60 per cent sucrose 4 1 and 0 3 ml of each was layered on polymerized acrylamide gel columns The acrylamide gel contained 5 per cent acrylamide and 0 1 per cent SDS The running buffer, 0 1 M sodium phosphate buffer was made in 0 1 per cent in SDS The lenghts of the columns varied in the different tests and accordingly the times of run, but a voltage of 50 V and current of 25 to 50 mA was used in each test After 2 to 8 hours the protein bands were fixed overnight with 20 per cent sulphosalicylic acid and stained with 0 25 per cent Coomasi blue for 6 hours The excess of stain was washed with 7 per cent acetic acid

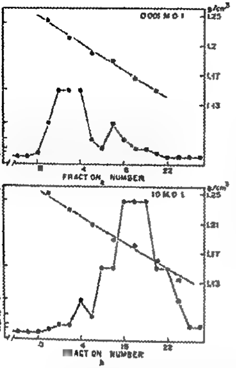


Fig 4 CsCl density gradient analysis of vesicular stomatitis virus haemagglutinin propagated with m.o.i. of 0.001 (a) and 10 (b) plaque forming units per cell. Linear density gradients were prepared from two solutions of CsCl with densities of 1.13 and 1.30 g/cm³. Centrifugation was for 16 hr at 35 000 r.p.m. in the Spinco SW 39 rotor. The buoyant densities for two haemagglutinins are 1.18 and 1.22 g/cm³ in both cases

centrated and purified as indicated above. Linear density gradients were prepared from two solutions of cesium chloride with densities of 1.13 and 1.30 g/cm³ and 0.6 ml of the haemagglutinin specimens was layered on top of 5.0 ml of these gradients. After centrifugation for 16 hours at 35 000 r.p.m. in the Spinco SW 39 rotor two visible bands were formed again in both preparations. Fractions of 4 drops each were immediately assayed for haemagglutination and density was measured in a refractometer (Fig 4). Two haemagglutinin peaks were formed in each of the two preparations obtained with different multiplicities of infection and these peaks were associated with the visible bands in the centrifuge tubes. In both cases the densities for two haemagglutinins were the same, 1.22 and 1.18 g/cm³.

Acrylamide Gel Electrophoresis

The upper bands and lower bands of separate centrifugation batches were pooled and adjusted to contain the same concentration of protein. They were then treated with SDS and 2-ME and were run in acrylamide gel electrophoresis. Fig 5 illustrates the results of one such experiment. The two haemagglutinating bands seemed to contain the same protein composition of 3 major proteins and 1 minor protein. However, the band of the smallest protein seemed to be fainter in the slower sedimenting band.

Electron Microscopy

The more rapidly sedimenting band almost uniformly contained whole virions only (Fig 6a) with clear surface projections. The electron micrographs of the more slowly sedimenting band showed only few complete virus particles but large numbers of particles with the length of one third of the virion and

Fig 5 Polyacrylamide gel electrophoresis of the purified haemagglutinins, the one sedimenting rapidly in sucrose gradient centrifugation on the left and the one sedimenting slowly on the right. Haemagglutinins were dissociated with 1 per cent sodium dodecyl sulphate and 1 per cent 2-mercaptoethanol at 100° C for 2 minutes and electrophoresed in 5 per cent polyacrylamide gel for 130 minutes at pH 7.0

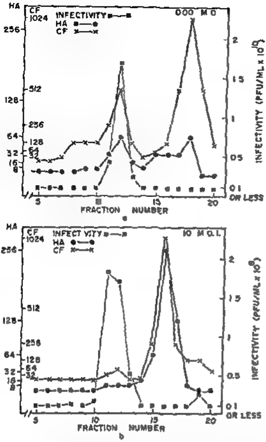


Fig 3 Sucrose gradient analysis of vesicular stomatitis virus propagated with m o i of 0 001 (a) and 10 (b) plaque forming units per cell showing the distribution of haemagglutinating complement fixing and infective activities Centrifugation on 15 to 45 per cent sucrose gradient was for 2 hr at 22 500 r p m *in the Spinco SW 25 rotor The scale* of infectivity is in (b) 1 $\log_{10}$ lower than in (a)

rapidly sedimenting haemagglutinin The positions of the bands in the gradient tubes suggested that these two haemagglutinins with different sedimenting properties represent infectious B and non infectious T particles of VSV With the aim of establishing this, haemagglutinin was produced with two different multiplicities of infection one of 0 001 (the standard system when producing haemagglutinin) and the other with 10 PFU cell Each culture was incubated for 36 hours to make the harvest conditions identical although with greater m o i the maximal amount of haemagglutinin may already have been reached in 16 to 20 hours After PFG precipitation and ultracentrifugation the antigen containing pellets were centrifuged in the sucrose gradient The same two bands as earlier were observed, but with the higher m o i the slower sedimenting upper band was now remarkably thicker than that obtained with the smaller m o i (Fig 2b) The HA, CF and infective activities are presented in Fig 3a With smaller m o i the two haemagglutinin patterns are approximately equal but with higher m o i the more rapidly sedimenting haemagglutinin has almost entirely disappeared and a very strong pattern of haemagglutinin was associated with the slower sedimenting band CF activity in the lower band also strongly decreased when the multiplicity of infection increased But it should be noticed that the CF pattern of the slower sedimenting band was not significantly *changed by increasing the multiplicity of in*fection With both multiplicities of infection about 90 per cent of the total infectivity was associated with the more rapidly sedimenting band The peak of infectivity and likewise the total infectivity, remained much lower with the higher m o i Because this haemagglutinin in the upper band may have been a product of disintegration during the concentration and purification processes VSV was *grown in the usual way from high* diluted seed virus for two days and the cells were sedimented by low speed centrifugation This unconcentrated, extracellular virus was immediately centrifuged in the sucrose gradient and the same two *haemagglutination* peaks were formed in the same fractions as always This indicated that the crude VSV antigens contained two haemagglutinins with different sedimentation properties A few *experiments* were also performed with New Jersey serotype and they suggested that this serotype also had two haemagglutinating *components* with different sedimentation properties and that their mutual proportions depended upon the input multiplicity of *infection*

Equilibrium Centrifugation in a CsCl Gradient

VSV haemagglutinins were produced with the m o i of 0 001 and 10 *PFU/cell con*

resembling so called T particles of VSV (Fig 6b) In addition there were some particles of various sizes which most nearly resembled the structures known as rosettes (2) In repeated experiments the lower band usually was packed with virions whereas in preparations of the upper band difficulties were encountered in finding viral material although the HA and CF titres of the two bands were approximately equal

DISCUSSION

In the present study haemagglutinin produced by diluted seed virus and a multicyclic growth was first precipitated with PEG, then eluted and pelleted again by ultracentrifugation, and finally layered on top of a sucrose gradient Two visible bands were constantly formed in the gradient tube and these two bands were always associated with two peak activities of haemagglutination These two haemagglutinins with different sedimentation properties could also be separated by equilibrium centrifugation in a cesium chloride gradient In our earlier experiments with VSV the only haemagglutinin found was associated with the virion (1) It is possible that, in the conditions used then (a very low m o i) only complete virions were formed

Experiments were performed to characterize this additional haemagglutinating component of VSV The results obtained here suggest that the more slowly sedimenting haemagglutinin was formed of T particles

According to many reports concerning the autointerfering T particles (4 5 6 8 12), it is probable that the crude antigens prepared in the present study also contained these particles The conclusion that the haemagglutinating capacity of the upper band was due to T particles was based on the following results

1) When the multiplicity of infection was increased the proportion of more slowly sedimenting haemagglutinin increased significantly The proportion of T particles is known to increase on such conditions (4 8)

2) In electron microscopy the upper band consisted mainly of T particles In addition there were small amounts of rosettes and structures that were probably consisting of unwound nucleoprotein Only whole virions bearing all surface structures could be seen in the band of more rapidly sedimenting haemagglutinin

3) In polyacrylamide gel electrophoresis the two haemagglutinins contained the same protein components three major and one minor protein, as described earlier (3 9 14 15) However, there was a slight difference in the quantity of the smallest protein component

4) There was no peak of infectivity in the additional haemagglutinating fraction

5) In cesium chloride centrifugation the two haemagglutinins could also be separated according to densities and the densities 1 18 for T and 1 22 g/cm^3 for B particles correspond rather well with the values reported earlier (1, 12) The slight difference in the quantities of the smallest protein in acrylamide gel electrophoresis may have been due to the fact that there were some rosettes and also nucleoprotein sedimenting in the same band with T particles and that the rosettes lack this protein The protein compositions of B and T should be identical (9 14)

The obvious and interesting thing is that the virions seem not to be as active haemagglutinins as T particles The lower band in the sucrose gradient with an infectivity titre of 10^9 PFU/ml was found to be packed with virions in electron microscopy However the HA titres were only $^1/_{16}$ to $^1/_{64}$ per 0 05 ml In the upper band on the other hand the haemagglutination titre may have been four times as high but is was sometimes difficult to find viral material in quantities sufficient for photographing Somewhat surprisingly the antigens with the highest haemagglutination titres were prepared with a high input multiplicity of infection and a growth of about 20 hours The antigens consisted mainly of T particles

It is actually not surprising that T particles also possess haemagglutinating capacity because they all have the same surface struc

Fig. 6 Electron micrographs of particles typical for the two haemagglutinin fractions with different sedimentation velocity showing (a) virions in rapidly sedimenting haemagglutinin and (b) more slowly sedimenting shorter particles resembling those of T particles. The line represents 100 nm

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SUPPRESSION OF CARDIAC ALLOGRAFT REJECTION IN ADULT RATS BY PRETREATMENT WITH BONE MARROW CELLS

Immunological Enhancement

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Rat hearts were transplanted across major histocompatibility loci in two donor recipient combinations A pronounced and specific suppression of rejection was obtained when recipients were treated with one injection of bone marrow cells 7 days prior to allografting Histological signs of immune reactions in the grafts were found and some rats produced circulating alloantibodies Skin grafts were rejected in the one combination but were accepted in the other, when applied to recipients with well tolerated hearts of the same genotype Normal response of recipient spleen cells in GVHR was obtained, and parabiotic union between recipient with functioning grafts and normal unmodified rats did not effect function of the transplants The suppressive effect of marrow pretreatment could not be transferred with serum taken from rats at the time of transplantation The possible mechanisms are discussed

The effect of a number of immunosuppressive drugs for the survival of heterotopic heart allotransplants in rats were studied by *van Bekkum Heystek & Marquet* (11) Pro longed survival time of a heart transplant in this experimental system was not as pron ounced as that of a kidney graft, this differ ence was interpreted primarily as a diversity in the susceptibility of the two kinds of tissues to immune attack

Attempts to produce antigen specific sup pression of the rejection response against a kidney allograft in rats have been reported (9, 10) and the most likely explanation given for the prolonged graft survival was that immunological enhancement had been in duced The present study was undertaken to 1) investigate the possibilities of prevention of heart allotransplant rejection by antigen pre treatment and 2) to parallel the finding of an enhancing effect of a single preceding pregnancy as observed in another study (6)

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MATERIAL AND METHODS

Rat strains Rats from inbred strains were used Wistar (Wi) males (250–400 gm) 3 5 months of age served as recipients The donors were Fischer (Fi) (Wi × Fi)F_1 hybrids and (Wi × BN)F_1 hybrids of both sexes aged approximately 10 weeks

Skin grafting Abdominal full thickness skin was

tures as the whole virion There is no evidence of whether or not the rosettes possess haemagglutinating activity In these experiments when PEG precipitated haemagglutinin was centrifuged for 90 minutes at 20 000 r p m in the Spinco 30 rotor some 20 per cent of haemagglutinin was constantly left in the supernatant Whether or not these are some aggregated surface projections or small rosettes cannot be concluded from the results presented Another interesting question is whether it is possible to produce actively haemagglutinating material by artificially disintegrating whole virions

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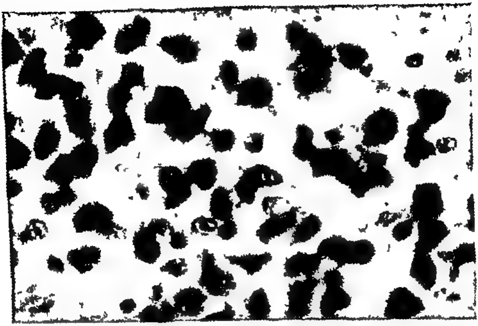

Fig 1 A cluster of pyroninophilic plasma cells is seen in a focus in a long term functioning heart allograft (Group 1) 60 days after transplantation Methyl green and pyronine (× 1000)

receiving serum were given the following course of treatment if not otherwise stated 1.5 ml intravenously immediately after heart transplantation 1.0 ml intraperitoneally at 24 48 and 72 hours after and continuing every other day till 1) rejection or 2) a total volume of 10 ml

Parabiotic union was performed between selected recipients and normal untreated syngeneic rats of same sex and of approximately same age This procedure was carried out under ether anaesthesia the incision emerging from the base of the ears to the back of the thigh Abdominal cavities were connected and the ventral and dorsal skin edges were sutured with 4 0 silk Plaster bandages were used to keep the rats in close contact during the first 10 days

EXPERIMENTAL DESIGN AND RESULTS

Group 1 is recorded in Table 1 Wistar rats received a single i v injection of 1×10^7 4×10^7 bone marrow cells from Fi or (Wi × Fi) F_1 rats After 7 8 days an allogeneic heart was trans

TABLE 3 *The Effect of Pretreatment with Donor Skin and Spleen Cells on Cardiac Allograft Survival*

Recipient strain	Pretreatment	Donor of heart skin and spleen	Heart survival days
Wi	1 skin graft	(Wi × BN)F_1	6
Wi	1 skin graft	(Wi × BN)F_1	7
Wi	1 skin graft	Fi	[illegible]
Wi	1 skin graft and 2×10^8 spleen cells i p	Fi	6
Wi	1 skin graft and 2×10^8 spleen cells i p	(Wi × Fi) F_1	6
Wi	1 skin graft and 2×10^8 spleen cells i p	(Wi × Fi) F_1	7

Unmodified Wistar recipient rejected (Wi × BN)F_1 hearts within 8.7 ± 1.5 days and (Wi × Fi)F_1 and Fi hearts within 9.4 ± 1.76 days (7)

TABLE 1 *The Effect of i v Injection of Bone Marrow Cells on Cardiac Allograft Survival Group I*

Reference number	Recipient	Bone marrow donor and dosis	Pretreatment interval days	Heart donor	Survival days
E 1	W_1	F_1 1 × 10	7	F_1	4
E 3	W_1	F_1 3 × [illegible]	8	F_1	191*
E 4	W_1	F_1 2 × 10	8	F_1	13
E 20	W_1	$(W_1 \times F_1)F_1$ 2 0 × 10^7	7	$(W_1 \times F_1)F_1$	142*
E 21	W_1	$(W_1 \times F_1)F_1$ 1 0 × 10^7	7	$(W_1 \times F_1)F_1$	142*
E 22	W_1	$(W_1 \times F_1)F_1$ 1 5 × 10	7	$(W_1 \times F_1)F_1$	142*
E 23	W_1	$(W_1 \times F_1)F_1$ 1 5 × 10	7	$(W_1 \times F_1)F_1$	142*
E 15	W_1	$(W_1 \times BN)F_1$ 2 × 10^7	7	F_1	11
E 32	W_1	$(W_1 \times BN)F_1$ 1 5 × 10^7	7	$(W_1 \times F_1)F_1$	9
E 33	W_1	$(W_1 \times BN)F_1$ 1 3 × 10^7	7	$(W_1 \times F_1)F_1$	9
E 34	W_1	$(W_1 \times BN)F_1$ 1 5 × 10^7	7	$(W_1 \times F_1)F_1$	10
E 35	W_1	$(W_1 \times BN)F_1$ 1 5 × 10^7	7	$(W_1 \times F_1)F_1$	12

* Still functioning

Unmodified Wistar recipient rejected F_1 and $(W_1 \times F_1)F_1$ hearts within 9 4 ± 1 76 (SD) days (7)

freed from fat and a transplant of 15 × 20 mm was sutured in place in a suprapannicular graft bed at the dorsum of the thorax Rejection was scored as the day on which the transplant was converted to a scab

Heart grafting was performed and survival times determined as described previously (5)

Bone Marrow was aspirated from the femur of etherkilled donor strain rats by the aid of a 21 gauge needle It was collected in normal saline and washed twice in Hanks balanced salt solution Following passage through a 25 gauge needle a counting of mononuclear cells in the suspension was performed

Bone marrow cells suspended in 1 5 ml volumes were injected intravenously in a tail vein or in a dorsal foot vein

Serological tests used were red cell haemagglutination and lymphocytotoxicity as described elsewhere (7)

Graft versus host reactions of recipient spleen cells were measured by the aid of the popliteal lymph node assay developed by *Ford et al* (4) The subcapsular kidney assay system described by *Elkins* was used in parallel in two recipients (3)

Serum transfer Blood from recipient rats was obtained from the tail vein or by aortic puncture Serum was stored at —20° C before transfer Rats

TABLE 2 *The Effect of i v Injection of Bone Marrow Cells on Cardiac Allograft Survival Group II*

Reference number	Recipient	Bone marrow donor and dosis	Pretreatment interval days	Heart donor	Survival days
E 5	W_1	$(W_1 \times BN)F_1$ 1 × 10^7	7	$(W_1 \times BN)F$	189*
F 8	W_1	$(W_1 \times BN)F_1$ 2 5 × 10	6	$(W_1 \times BN)F_1$	22
F 9	W_1	$(W_1 \times BN)F_1$ 1 2 × 10^7	7	$(W_1 \times BN)F_1$	188*
E 18	W_1	$W_1 \times BN$ F_1 1 × 10	7	$(W_1 \times BN)F$	144*
E 19	W_1	$(W_1 \times BN$ F_1 1 10	[illegible]	$(W_1 \times BN)F$	20
E 37	W_1	$(W_1 \times BN$ F 1 5 × 10^7	7	$(W_1 \times BN)F$	57*
F 38	W_1	W_1 BN F_1 1 [illegible] × 10	7	$(W_1 \times BN)F_1$	19*
E 39	W_1	$W_1 \times BN$ F 1 3 × 10	7	$(W_1 \times BN)F_1$	49*
F 40	W_1	$(W_1 \times BN)F_1$ 1 [illegible] × 10	7	$(W_1 \times BN)F_1$	49*
F 41	W_1	$W_1 \times BN$ F_1 1 3 × 10	7	BN	33
F 10	W_1	F_1 3 3 × 10^7	7	$(W_1 \times BN)F_1$	7
E 11	W_1	F_1 0 7 × [illegible]	7	$(W_1 \times BN)F_1$	9
F 12	W_1	F_1 2 0 × 10	7	$(W_1 \times BN)F_1$	7

* Still functioning

Unmodified Wistar recipient rejected $(W_1 \times BN)F_1$ hearts within 8 7 ± 1 75 (SD) days 7

planted to these recipients Donor hearts of same genotype as the initial cell inoculum enjoyed prolonged and probably indefinite survival in most recipients, in contrast to this were hearts transplanted to third party pretreated recipients rejected acutely within 10 days

Group II is recorded in Table 2 Wistar rats pretreated with 1 5 3 0 × 10^7 bone marrow cells from (Wi × BN)F_1 7 days before heart transplantation did not reject hearts of the bone marrow cell donor genotype whereas third party pretreated rats rejected acutely

The heart transplants in groups I and II that survived for prolonged periods occasionally suffered a rejection crisis from approximately day 7 till around the 21st day after transplantation During this period, increase in tension of the graft and decrease in potency of the beat was noted Having survived throughout this period hearts regained softness became smaller and beatings through the skin could be seen again No clinical alterations were found during the following periods up till ■ months

Table 3 gives the results of heart transplant survival in recipients which were pre immunized by skin grafts and injection of large numbers of spleen cells from the respective donor strain The pre immunization took place from 1–6 weeks before heart allografting was performed It was evident that acute rejection took place following these regimens and that rejection times were within the lower limit of the control range

The results of serological analysis of recipients with long term functioning grafts are presented in Table 4

It appears that antibodies of both classes are produced in some recipients and that graft survival times are independent of the presence or absence of these antibodies

Histological examinations of long term surviving hearts which were biopsied or removed while functioning excellently showed focal infiltration of typical mature plasmacells with pronounced cytoplasmatic pyroninophilia (Fig 1) Endothelial proliferation in vessels and of endocardium was occasionally present (Fig 2) Apart from this the grafted tissue was morphologically normal as

Fig 2 An artery in a well functioning heart transplant 60 days after transplantation shows intimal thickening and cell proliferation Haematoxylin and eosin (× 400)

compared to isografted hearts removed after some time

Skin transplants originating from (Wi × BN)F_1 were applied instead of hearts to two recipients in group II on the 7th day after treatment with 1 5 × 10 bone marrow cells i v

The skin grafts were rejected within 14 days which is normal in unmodified recipients

Recipients carrying well established long term functioning heart transplants were challenged with donor strain skin and with third party skin (Table 4) Two recipients tested in Group I rejected both their skin grafts acutely (< 14 days) without registrable effect on the functioning heart transplant In group II recipients tested rejected third party skin acutely while donor strain skin (Wi × BN)F_1 has survived in good condition for over 4 months with no obvious adverse effect on the heart transplant One of these (E 9) has continued to produce agglutinating and cytotoxic antibodies of titres up to 1 32 for almost 6 months

Parabiosis was performed between one recipient in each of the groups and a normal male rat Both pairs survived the procedure and after 41 days and 65 days heart transplant are beating excellently and a black (Wi × BN)F skin graft on the one recipient ■ surviving in good condition Serological investigations of the parabiotic partners have shown that cytotoxic antibodies against the

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□ Haemagglutinins which are scored in the usual way (+ to ++++)

■ Lymphocytotoxic antibodies which are scored from + — ++++ corresponding to a percentage kill from 20 per cent to 100 per cent

× The day of observation

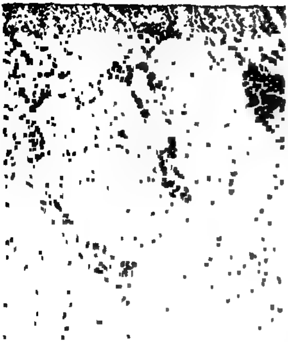

Fig 3 The left kidney of a $(W_1 \times F_1)F_2$ rat received a subcapsular injection of 5×10^7 spleen cells originating from a W_1 with a well-tolerated $(W_1 \times F_1)F_2$ heart transplant (E 20) The photograph shows the invasive destructive graft versus host reaction which was seen deep in the cortex of the kidney 7 days after injection GVHR a m Elkins (3) Haematoxylin and eosin (× 250)

transplant donor strain had been developed and were of equal titre in both

Spleen cells from rats pretreated with bone marrow cells (1 5 × 10) 7 days earlier, tested for *GVH reactivity* in a semiquantitative system (Fig 3) and in a quantitative assay system (Table 5) showed a reactivity not significantly different from that of control rats Spleen cells taken from rats with long tolerated heart and skin grafts tended to give an increased GVHR but not statistically significant (Wilcoxon test)

The results obtained upon treatment of unmodified heart transplanted rat recipients with serum originating from rats pretreated 7 and 12 days earlier with bone marrow cells are shown in Table 6

Significant retardation or prevention of rejection was not found One rat, however, treated with serum drawn from a recipient with a well established long term heart transplant (group II) has not rejected after 137 days which is a significantly prolonged survival time This rat received a total of 10 ml serum drawn from rat No E 18 (haem agglutinating titre 1 8)

DISCUSSION

The two heart allotransplantation systems used employ rat strains differing at the major histocompatibility (Ag B) locus and these have been analysed earlier in respect to graft survival, histology and serology in unmodified recipients (7) The results obtained following bone marrow cell injection in the dose range and with the time interval used in the present study, clearly indicate that rejection of a heart allograft was effectively prevented by this treatment in the majority of recipients most pronounced in cases with heterozygous donors In contrast pre-immunization by skin grafts and great numbers of spleen cells tended to accelerate the rejection process and indicates that the mode and severity of sensitization is of decisive importance for the immune reaction in the subsequent period

The acute rejection reactions to occur in

TABLE 5 *Graft Versus Host Assay*

Dose of spleen cells in foot of $(W_1 \times BN)F_1$	Mean weight of popliteal lymphnode in milligrams			
	Control $(W_1 \times BN)F_1$	Control W_1	E 37 day 7	E 18 day 105
0 05 × 10	2 6 (6)	7 9 (10)	5 0 (4	10 6 5)
0 2 × 10	2 5 (4	18 4 (8)	9 5 (4)	21) 6)
0 8 × 10	3 3 (6)	49 2 (10	35 3 (6	5 3 3)
2 0 × 10	4 4 (10)	78 3 ([illegible]	61 2 (1)	86 3)

The figures in parentheses represent number of lymph node estimates

$(W_1 \times BN)F_1$ host rats were 5 7 weeks of age and injected with 0 15 ml volumes subcutaneously in the foot 7 days prior to lymph node harvest

TABLE 6 *Serum Transfer Experiments*

Recipient strain	Serum treatment: Total volume injected ml	Serum treatment: Source of serum	Donor of heart and bone marrow	Heart survival days
W_1	1 8*	7 days after bone marrow	F_1	8
W_1	1 7*	7 days after bone marrow	$(W_1 \times F_1)F_1$	8
W_1	7 3	7 days after bone marrow	$(W_1 \times F_1)F_1$	12
W_1	7 3	7 days after bone marrow	$(W_1 \times F_1)F_1$	12
W_1	5 2	7 days after bone marrow	$(W_1 \times F_1)F_1$	11
W_1	7 7	12 days after bone marrow	$(W_1 \times F_1)F_1$	12
W_1	5 3	7 days after bone marrow	$(W_1 \times BN)F_1$	6
W_1	7 8	7 days after bone marrow	$(W_1 \times BN)F_1$	8
W_1	10 0	From recipient with long term functioning heart transplant	$(W_1 \times BN)F_1$	137§

* Total serum volume injected at the day of transplantation The general course of serum treatment is described in Materials and Methods
§ Still functioning

recipients pretreated with bone marrow cells from one strain and transplanted with hearts from another serve as controls that an immunologically specific effect has been produced

The uniform results in the two parallel groups have been taken as an indication that the phenomenon observed is not a coincidence restricted to one special donor recipient combination

The mechanisms responsible for the prevention of acute rejection could be A) actively acquired tolerance (2) or B) active immunological enhancement (1 8) or both alternating If A) is the explanation it must be considered to be a partial tolerance as skin grafts applied instead of hearts were rejected with normal promptitude and because histological signs of reaction were found The development of relatively high titred transplantation antibodies in some recipients at early stages as well as for prolonged periods in some cases tends to exclude the possibility of tolerance induction in these rats a feature which is further substantiated when the normal results of GVHR and the failure of parabiosis to induce rejection are taken into consideration

Immunological enhancement is an immunologic phenomenon dependent on specific alloantibody resulting in prolongation of survival of an incompatible graft Enhancement was originally defined in tumour graft systems (8), but the terms and conditions have been used during the last years in the field of organ allografting One of the main points in immunological enhancement is the possibility of passive transfer of the phenomenon of prolonged graft survival to untreated recipients by means of serum antibody

In this study, the failure of serum taken on the day of heart transplantation from marrow treated rats to prolong graft survival in new recipients indicates that enhancing antibodies of 'clinical significance' are not preformed in recipients at the time of transplantation When an additional cardiac allograft was performed enhancement which could be passively transferred would be produced This finding needs further confirmation and an analysis of the 'enhancement antibody stimulating' mechanism is in progress

A hypothesis which can be advanced is the existence in organ allgrafting systems of a phenomenon which could be termed immunological enhancement at the cellular level characterized by non serum transferable antigen specific suppression of rejection

at a time when immunocompetent cells retain full reactivity, when tested outside the recipient If so, the mechanisms responsible should be cellular production of contrasensitizing (blocking) factors which are bound to antigens of the transplant and eventually to receptors on immunocompetent cells in the recipient If so, the phenomenon of prolonged graft acceptance should be transferable by means of recipient lymfoid cells This will be tested in further experiments in this laboratory

The reason for the "take' of late skin grafts in the group employing $(W_1 \times BN)F_1$ donors and rejection in the $(W_1 \times F_1)$ group is not clear at present It could be interpreted as an enhancement more effective in the one combination than in the other or that skin specific antigens not represented in bone marrow and heart, might be present in one and not in the other donor strain

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SELECTIVE IMMUNOGLOBULIN DEFICIENCY IN CATTLE AND SUSCEPTIBILITY TO INFECTION

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Sera from animals, mainly of the Red Danish Milkbreed, have been examined by immunochemical analyses in order to select possible IgG-2 immunoglobulin deficiencies According to previous findings a low frequency was found in the healthy animal material (1 out of 417 animals) whereas the frequency of this immunoglobulin deficiency was strikingly much higher among diseased animals, especially among animals with pyogenic infections (13 out of 93 animals) It was assumed that lack of IgG 2 may be associated with a reduced resistance to infections, in particular those caused by pyogenic bacteria

Four classes of immunoglobulins have been identified in cattle IgG-1 and IgG-2 have been described and characterized in detail by *Murphy et al* (1965), *Pierce and Feinstein* (1965) and *Kickhöfen et al* (1968). The existence of IgM (*Murphy et al* 1965) and IgA (*Mach et al* 1969, *Vaerman* 1970) have likewise been demonstrated A series of experiments, most recently by *Klaus et al* (1969), have shown that IgG-1 is selectively transferred from serum to colostrum Hence, the post-suckling calf serum usually contains substantial quantities of this immunoglobulin class, passively derived from the dam Gradually, this maternally derived immunoglobulin is catabolized and successively the synthesis of all classes of immunoglobulins is initiated under the influence of environmental antigenic stimulation and increasing immunological competence Consequently, immunoglobulin deficiency may be based upon one or more of the following circumstances 1) Lack of maternal immunoglobulin in the new-born, 2) failure of the young animal to initiate synthesis and 3) discontinuation of an existing synthesis While much attention has been attached to neonatal immunoglobulin deficiency secondary to inadequate colostral intake or failure to absorb immunoglobulin from the gut, there seems to be only scanty information available on lack of immunoglobulins in adult cattle

Mansa (1965) described a selective immunoglobulin deficiency in normal adult cattle of the Red Danish Milkbreed By immunoelectrophoretic analysis it was found that 8 out of 780 adult animals apparently lacked an immunoglobulin component, designated 7S-gamma When comparison is made with studies by *Murphy et al* (1965) and *Aalund* (1968) it appears that Mansa's 7S-gamma globulin is identical with IgG-2 Furthermore, lack of immunoglobulin in adult cattle has been reported from Central Africa (*Protost*

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et al 1965) By immunoelectrophoresis it was demonstrated that a small but not precisely determined number of animals lacked a cathodically migrating immunoglobulin which presumably corresponded to IgG-2

In a recent study comprising 29 adult cattle patients with various diseases, including bacterial and parasitic infections 5 animals (17 per cent) were deficient of IgG-2 (*Nansen* 1970) This contrasted with the finding that only 4 out of 180 normal sera (appr 2 per cent) lacked this immunoglobulin This apparent accumulation of immunoglobulin deficiency among diseased animals has been examined more closely in the present investigation

MATERIALS

Healthy Animals

417 sera were obtained from animals originating from two different geographical areas

a) During the years 1968–1969, 320 sera were obtained from normal female animals from eastern and southern regions of Jutland Ages of the animals ranged from 3 to 12 years, the average age being 6 years Distribution of animals on breeds was as follows 21 per cent belonged to the Black Pied Danish Milkbreed (SDM), 59 per cent belonged to the Red Danish Milkbreed (RDM), 19 per cent were Jerseys and 1 per cent were cross breeds

b) In 1968 serum samples were obtained from 97 normal female animals from various herds of Sealand Ages ranged from 1 to 9 years, averaging 4 years There were 93 per cent RDM s and 7 per cent Jerseys

Diseased Animals

312 sera, including the above mentioned 29 sera (*Nansen* 1970), were obtained from patients housed in the Medical Clinic for Large Domestic Animals during the years 1967 1969 The majority of animals originated from herds at Sealand The patient material of the clinic does not reflect entirely the disease situation in the area of Sealand as a whole, since most admitted animals suffer from chronic diseases with a rather high lethality At approximately monthly intervals serum was drawn routinely from all hospitalized animals more than six months old

The collected samples were not representative of the entire patient material, since animals with acute, fatal diseases or animals with mild easily curable diseases stayed in the clinic for only short periods and consequently were not always subject to the routine blood sampling The collected sera originated from appr 50 per cent of the patients in the age group ½ to 9 years The average age was 5 years Seventyfive per cent were RDM s 13 per cent were Jerseys 8 per cent were SDM s and 4 per cent of the animals were other breeds or crossbreeds The vast majority of animals were females Thus, there were only 13 bulls (11 RDM s 1 SDM and 1 crossbreed) According to the aetiology of the disease the animals could be classified into 4 groups A 100 animals with non infectious diseases like, *e g* intoxications nutritional and metabolic disorders, B 89 animals with viral, parasitic or bacterial diseases other than pyogenic bacterial infections C 30 animals with pyogenic bacterial infections secondary to traumatic lesions, *e g* traumatic reticuloperitonitis teat lesions, surgical incisions etc, D 93 animals with primary pyogenic bacterial infections *e g* purulent bronchopneumonia mastitis, endometritis pyaemia

The sera were stored in small aliquots at minus 20° C until immunoelectrophoretic analysis

METHODS

All sera were screened by immunoelectrophoresis using the micromethod of *Scheidegger* (1955) Antisera were obtained from rabbits immunized with pooled serum from 22 normal animals (RDM) Examination of serial dilutions of IgG 2 demonstrated that visible precipitates were formed in immunoelectrophoresis with IgG 2 concentrations down to approximately 180 µg/ml When no IgG 2 precipitates were formed in the immunoelectropherograms the sera were further analyzed by single radial immunodiffusion according to the method of *Mancini et al* (1965) as slightly modified by *Jensen* (1966) The antiserum imbedded in the agar gel was obtained by immunizing rabbits with IgG 2 isolated from one serum (RDM) by anion exchange chromatography on DEAE Sephadex Monospecificity was achieved by absorption of the rabbit antiserum with IgG 2 deficient serum. Application of serial dilutions of IgG 2 to 4 different agar plates revealed that the lowest concentration which produced visible precipitates was 6 µg/ml This limit could not be lowered further since a reduction of the antibody content in the agar produced indistinct precipitates Thus the present procedure would distinguish sera with IgG 2 concentrations below 6 µg ml from sera with IgG 2 levels in the range of 6 to 180 µg/ml

RESULTS

Healthy Animals

Among the 320 animals from the Jutland area, one RDM cow (6 years) was deficient

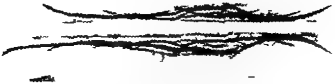

Fig 1 Immunoelectropherogram of an IgG 2 deficient serum, J No 375/67 (top well) and a normal serum (bottom well) precipitated with anti bovine serum (antibody trench) Cathode to the left

of IgG 2 as evidenced by immunoelectrophoresis and single radial immunodiffusion This animal was clinically normal and presented no history of *e g* recurrent infections All animals from the Sealand area contained IgG 2 as detected by immunoelectrophoresis

Diseased Animals

Among the 312 patients examined, IgG 2 could not be demonstrated by immunoelectrophoresis (Fig 1) in 22 female animals (7 per cent) All these animals belonged to the Red Danish Milkbreed Ages and main diagnoses are listed in Table 1 The IgG 2 deficiency was very unequally distributed among the 4 major disease groups Thus, only one out of 100 animals of the non infectious group A was IgG 2 deficient (1 per cent) while 5 out of 89 animals of group B (6 per cent) and 3 out of 30 animals of group C (10 per cent) lacked this immunoglobulin The highest number of IgG 2 deficient animals was found in the group of animals with pyogenic infections (group D) i e 13 out of 93 animals (14 per cent) In only 4 animals, one of group A two of group C and one of group D, could IgG 2 be demonstrated by the more sensitive single radial immunodiffusion analysis (see Table 1) Thus, the majority of the selected immunoglobulin deficient sera contained less than 6 μg IgG 2 per ml Ages of the IgG 2 deficient animals (average 3 years) were somewhat lower than those of animals in the entire patient material (average age 5 years) While the IgG 2 deficient animals of groups A B and C were admitted to the clinic with very different anamneses the immunoglobulin deficient animals of group D seemed to share a more characteristic disease history

TABLE 1 *Ages and Diagnoses of 22 IgG 2 Deficient Female Animals of the Red Danish Milkbreed*

Animal J No	Age (years)	Diagnosis	Disease group
138/66	2	Abomasoenteritis catarrhalis	B
187/66	2½	Bronchopneumonia purulenta et necroticans	D
217/66	5	Enteritis paratuberculosa	B
306/66	2	Peritonitis fibrosa et purulenta traumatica	C
314/66§	2	Bronchopneumonia purulenta	D
374/66	1	Bronchopneumonia purulenta apostematosa	D
389/66	3	Pyaemia	D
420/66	3	Bronchopneumonia purulenta	D
449/66§	3	Encephalomalacia	A
60/67	1	Abomasitis hyperplastica verminosa	B
189/67	[illegible]	Bronchopneumonia purulenta Lymphocytosis	D
194/67	3	Abomasoenteritis catarrhalis	B
274/67	5–7	Mastitis purulenta	D
278/67§	3	Peritonitis purulenta traumatica	C
290/67	6–7	Pyaemia	D
375/67	2	Fascioliasis	B
398/67	[illegible]	Bronchopneumonia purulenta	D
446/67	2½	Pyaemia	D
59/68	2½	Bronchopneumonia purulenta	D
420/68	3	Pyaemia	D
423/68	2½	Bronchopneumonia purulenta	D
487/68§	2½	Peritonitis purulenta traumatica	C

§ IgG 2 demonstrable by the subsequent radial immunodiffusion analysis

4*

Before these animals arrived to the clinic there had usually been periods of repeated attacks of infection the first of which often coincided with the postparturient period The infections might be temporarily controlled by antibiotics but recurred after cessation of treatment The same pattern was established during the stay in the clinic where the general condition of the animals usually would be very poor The majority of animals were killed after some weeks of observation *Corynebacterium pyogenes* was the bacterium most frequently isolated Also *Streptococcus* and *Micrococcus pyogenes* were isolated from some animals It is noticeable that the formol gel reaction was negative in 7 out of the 13 IgG 2 deficient animals with pyogenic infections

Finally, it should be mentioned that IgG-1 was always demonstrable by immunoelectrophoresis both in normal and diseased animals IgM and IgA precipitation lines, which may sometimes be difficult to recognize and identify in the immunoelectropherograms were not evaluated

DISCUSSION

The lack of detectable serum IgG 2 is assumed to be due to an extremely reduced or even failing synthesis, and not to excessive catabolism (*Nansen* 1970) If an individual is not synthesizing a protein present in other members of the same species e g due to lacking genetic information immunization with this protein may induce the synthesis of iso antibodies Conversely, such iso-antibodies may be indirect proof of a complete and genuine lack of the protein concerned *Gahne* (1964) was able to develop iso antibodies against bovine alpha 2 macroglobulin by injecting normal serum into a cow with an inherited lack of this protein Similarly, attention should be focused on the possible production of iso-antibodies against bovine IgG 2 It should be emphasized that a genetic basis for many dysimmunoglobulinaemic conditions of man has been demonstrated and the question arises whether lack of IgG 2 in cattle might have a similar background Unfortunately available information about this very important point is still insufficient and inconclusive The distribution between males and females should be closely examined in this context IgG 2 deficient bulls were not found in the present study comprising 13 bulls and 280 sera from healthy adult bulls (RDM s mainly) all contained IgG 2 (*Mansa* 1970) A larger number of males should be examined to reveal possible IgG 2 deficiencies

Observations by the author (*Nansen* 1970) and by *Aalund and Kruse* (1971) seem to indicate the existence of at least two antigenic entities within IgG 2 Presumably not all sera contain the entire IgG 2 antigenic spectrum Since IgG 2 used for immunization in the present study was isolated from one serum only, the rabbit antiserum might be incomplete in the sense that it could not detect all IgG 2 antigenic determinants The antiserum used for the initial immunoelectrophoretic screening, on the other hand was developed by immunizing rabbits with a pool of serum from several animals Sera which did not develop IgG 2 precipitates with this antiserum were submitted to immunoquantitation in agar gel containing the above mentioned anti IgG 2 It should therefore be emphasized that IgG 2 components which might remain unrecognized with the used anti IgG 2 occurred at concentrations too low to be detected by immunoelectrophoresis using a more complete antiserum

The most conspicuous finding in the present study was the uneven distribution of non detectable levels of IgG 2 between normal sera and sera obtained from diseased animals The low frequency found in the normal material (1 out of 417 animals) corresponded to previous findings (*Mansa* 1965 1970 *Nansen* 1970) whereas the frequency among the diseased animals was strikingly much higher especially among animals with pyogenic infections This leads to the assumption that lack of IgG 2 is associated with a reduced resistance to certain infections especially to those caused by pyogenic

bacteria. A particular role of IgG-2 in the defence against pyogenic bacteria could be expected. Thus these infections are usually associated with an increased production of especially IgG 2 in animals with normal ability to synthesize immunoglobulins (*Nansen* 1970), and generally the course of infections in the latter animals was not as severe as that in IgG 2 deficient animals. An alternative possibility is that the hospitalized animals have predominantly been admitted from certain cattle herds or families with a genetically determined high frequency of non-detectable levels of IgG-2. However, this could only play a minor role if any since 1) the animals originated from a variety of herds in different areas of Sealand and 2) no more than one IgG-2 deficient animal was admitted from any herd and 3) all 100 patients but one with non-infectious diseases (group A) contained IgG 2 in their sera.

Immunoglobulin deficiency syndromes in man have been classified into several groups, most of which can be recognized only when the individual classes or subclasses can be identified, *e. g.* by immunoelectrophoresis or specific immunoquantitation. In case the synthesis of one or possibly two immunoglobulin classes is disturbed the term dysimmunoglobulinaemia has been proposed (*Zawadski & Edwards* 1967) and lack of IgG 2 in cattle can be designated accordingly. Numerous dysimmunoglobulinaemias due to failing synthesis in man have been described (*Hobbs et al.* 1967, *Pelkonen* 1969, *Radl* 1970). They constitute a heterogeneous group of syndromes designated primarily by the class or classes of immunoglobulins involved. It appears that most selective immunoglobulin deficiencies in man are associated with decreased resistance to infections. In addition many of them are associated with various disorders such as defects of the thymus system, malabsorption syndromes and ataxia telangiectasias. Apparently the lack of bovine IgG 2 was primarily characterized by decreased resistance to infections and not associated with any obvious syndromes. Although the immunoglobulin deficiency in the bovine is selective, it offers clinical points of similarity with the congenital, sex linked, broadbanded hypoimmunoglobulinaemia in man, usually referred to as the "Bruton type" (*Bruton* 1952). Thus a typical feature of this syndrome is the reduced resistance to pyogenic bacteria such as micrococci, streptococci and meningococci. Usually the first symptoms are seen at ages of 6 months to 3 years, which is comparable with the observation in the present study that IgG 2 deficient patients were relatively young animals. Lung infections are most frequently observed in the "Bruton type" of hypoimmunoglobulinaemia, but otitis, sinusitis and septicaemia are often encountered. Like in IgG 2 deficient cattle the infections may be temporarily controlled by antibiotics, but recurrence is a nearly unavoidable feature. On the other hand development of viral infections are apparently not more severe than those in normal individuals. This appears to be in accordance with findings in studies by *Provost et al.* (1965) who observed that immunoglobulin (IgG 2 ?) deficient cattle in Central Africa were not more susceptible than normal animals to viral diseases such as Rinderpest despite the fact that the immunoglobulin deficiency coincided with a lack of *in vitro* detectable specific antibodies. Finally it should be noted that Leptospira agglutinins were found to be evenly distributed among normal and IgG 2 deficient sera of the Red Danish Milkbreed (*Mansa* 1965).

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COMPARISON OF NUMERICAL PROCEDURES FOR GROUPING PSEUDOMONAS BACTERIOPHAGES ACCORDING TO LYTIC SPECTRA

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The host ranges of 113 typing bacteriophages have been studied at their Routine Test Dilution (RTD) for 486 strains of *Pseudomonas aeruginosa* The resulting data matrix has been analysed by numerical allocating procedures employed in numerical taxonomy The Jaccard Sneath similarity index (S_{JS}) and the simple matching coefficient (S_{SM}) were calculated and the results of the weighted and the unweighted pair group average cluster analysis (WPGA and UWPGA) compared The S_{SM} seemed to produce misleading results Phages that lysed less than 15 per cent of the bacterial strains tended to be grouped together at high similarity levels regardless of real differences in their host ranges For such phages there was a linear relationship between per cent lysis and S_{SM} The best representation of the similarity index was obtained with the UWPGA applied to the S_{JS} matrix ([S_{JS}] UWPGA) This rendered a cophenetic correlation coefficient of 0 9303 in contrast to r = 0 8933 with the [S_{JS}] WPGA

Since 1960 nine different bacteriophage typing sets have been introduced for *Pseudomonas aeruginosa* (9 15 16 24 25, 27 28 33 34, 45 47) The simultaneous existence of several non related sets seemed undesirable the chances were that by proper selection among these very sets one might succeed in constructing a new set which more closely fulfilled properties desirable for a bacteriophage typing set (1) Little is known however about the concordance of lytic action for the phages involved, since the sets were constructed by conventional procedures

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An efficient and objective scanning of a large body of data is possible by numerical procedures Computational procedures for classification have rendered particularly valuable contributions to taxonomy around which a whole new branch numerical taxonomy has emerged (43) Similar procedures have demonstrated their usefulness in fields like psychology (37) ecology (40) in the classification of broches (14) and in the comparison of chemicals (7 12) Since each bacteriophage reaction on a set of bacteria must be equally valuable in defining the host range for each bacteriophage the Adansonian principle of equal character weighting applies Consequently selected procedures for numerical allocation reviewed by Sokal & Sneath

TABLE 1 *Pseudomonas aeruginosa Typing Bacteriophages Investigated, Their Designation and Derivation*

Designations: Used in this study	Other	Obtained from
1 21	2-7A-7B-16-21A-21B-24 31A-31B-44-68-73-109-119x 354-1214 F7-F8-F10-M4-M6	RBL
22-24	Col 11 Col 18 Col 21	EA
25	Pc	CGM
26-39	1-2-3A-3B-4-5 6-7 8 9 10-11-12-13	EM
40-43	3-11-95-113	JBG
44-49	B3 F116-D3C L*(1)-D3C L*(2)-E79-G101	BWH
50-52	103g 176p-188/I	JDP
53-56	Px2-Px3 Px5 Px7	RHO
57-67	2A-2B-3-4-5 6-8-9 10-12A-12B	EK
68-89	Clc Clt C3 C4 C7-C9-C13-C15-C16 C19 C21-C22 H95 H116-H249 P2 P6-P7 P8 P9 P10-P12	VLS
90-113	I IIIA IV V VI VII-IX-X-XI XII-XIIIA-XIIIB-XVI 1-2 3-6 9 16-18 19-20 21-22	HCZ

EA = Dr *Elisabeth Asheshov* and Dr *M T Parker* Cross Infection Reference Laboratory Colindale London England

JBG = Dr *J B Grogan* University Medical Center Jackson Miss USA

BWH – Dr *B W Holloway*, Department of Genetics Monash University Clayton Victoria Australia

EK = Dr *E Kaklamanis* 8 Karneadou Street Athens Greece

RBL = Dr *R* [redacted] *Lindberg* US Army Surgical Research Unit Brooke Army Medical Center Fort Sam Houston Texas USA

CGM = Mr *C G Mason* Biological Sciences Boots Pure Drug Company Nottingham England

EM – Dr *Eugenia Meitert* Institutul de Microbiologie Parazitologie [redacted] Epidemiologie Bucurest Romania

RHO = Dr *R H Olsen*, Department of Microbiology University of Michigan Ann Arbor Michigan USA

JDP = Dr *J D Piguet*, Service de microbiologie médicale Institut d hygiene Geneva Switzerland

VLS – Dr *Vera L Sutter* Veterans Administration Center Los Angeles California USA

HCZ Dr *H C Zanen* Streeklaboratorium Volksgezondheit Arnheim *Holland*

(43), should be particularly suitable for demonstrating differences and similarities within a large body of typing phages

It appears that previous bacteriophage typing sets, be it for pseudomonas, staphylococci, coli, salmonella, or mycobacteria have evolved mainly from subjective selection This, for a successful outcome, implies an intuitive, repetitous process of meticulous trial and error Certainly, the end result may be perfectly satisfactory as the international phage typing set for staphylococci amply demonstrates But in the process, unique phages may more easily be passed by or less suitable ones be included The selection of the proper combination of phages for a typing set may be achieved through numerical allocating procedures applied to the lytic spectra of the phages This would render an indication of what phages are different or similar in their ability to attack bacteria

However, a whole array of numerical allocating procedures are available Such procedures have been applied to xanthomonas bacteriophage lytic spectra by *Colwell et al* (5) An initial comparison and testing of techniques appeared essential From the experience reported in the literature the Jaccard-Sneath similarity index (S_{JS}) and the simple matching coefficient (S_{SM}) (43) were preferred as similarity indices The pair group clustering methods using simple averages were chosen to achieve group structure

The purpose of this paper is to elucidate the reliability and pitfalls of two coefficients of association and two clustering procedures for the grouping of bacteriophages according to their lytic spectra

MATERIALS AND METHODS

Strains

The 113 typing bacteriophages are presented in Table 1 The 486 strains of *Pseudomonas aerugino*

TABLE 2 *Origin of the 486 Strains of Pseudomonas aeruginosa Employed*

Number of strains	Source and contributors
9	Dr *R G Doggett*, Texas Institute for Rehabilitation and Research, Texas Medical Center, Houston, Texas, USA (mucoid strains).
15	Dr *J Govan*, Department of Microbiology, University of Edinburgh, Scotland (pyocine typing indicator strains)
12	Dr *B W Holloway*, School of Microbiology, University of Melbourne, Parkville, Victoria, Australia
4	Dr *J Y Homma*, Institute of Infectious Diseases, University of Tokyo, Tokyo, Japan (serotype strains)
12	Dr *H Kleinmaier*, Hygiene-Institut der Universität Heidelberg Heidelberg W Germany (serotype strains)
13	Dr *E J L Lowbury*, MRC Research Unit, Birmingham Accident Hospital, Birmingham, England
1	National Collection of Type Cultures, Colindale London England (NCTC 6749)
13	Dr *M T Parker*, Cross Infection Reference Laboratory, Colindale, London, England (12 pyocine indicator strains and 1 serotype strain)
2	Dr *O Sandvik*, The Veterinary College of Norway, Oslo Norway (one is a serotype reference strain)
27	Dr *B Thom*, Cross Infection Reference Laboratory, Colindale, London, England (includes 5 mucoid strains)
6	Dr *M Veron*, Institut Pasteur, Paris (serotype strains)
87	Bacteriophage propagating strains, see Table 1
1	Microbiological Department, Haukeland sykehus, Bergen, Norway
284	From hospitalized patients of this hospital, Rikshospitalet Oslo Norway
486	Total

ia used are listed in Table 2 All bacterial strains were freeze dried immediately after reception, the phages after their first propagation and purification

Purification, propagation media, and typing method are described in a later report (1)

Numerical and Statistical Procedures

Of all the existing permutations of similarity indices, the Jaccard Sneath (S_{JS}) (19, 38) and the simple matching coefficient (S_{SM}) (41) at the outset were regarded as the potentially most useful These are defined as

$$S_{JS} = \frac{n_{JK}}{n_{JK} + n_{jK} + n_{Jk}} \text{ and } S_{SM} = \frac{n_{JK} + n_{jk}}{n}$$

where the notations denote the constituents of the ONU pair contingency table according to (43) such that n is the total number of reactions, the capital subscripts indicate positive, and j and k the negative reactions respectively for ONU's 'J' and 'K' In this connection, Routine Test Dilution (RTD) scores of ± (3) and less were indicated by the digit 0 and the + and the ++ scores by the digit 1 The 486 bacterial strains constitute the characters for the Q matrix upon the phages No feature was either all positive or all negative for the entire phage set For this study, the data matrix was accepted with the knowledge that typing reactions may vary from typing to typing (2)

On each of the two similarity matrices, S_{JS} and S_{SM}, two distinct sorting procedures were compared, the weighted and the unweighted pair group average cluster analysis (WPGA and UWPGA) of *Sokal & Michener* (41) Not considering factor analysis, these were regarded the potentially most powerful allocating procedures for this problem Simple averages have been computed for each clustering cycle (43) These procedures have been programmed in Fortran IV language by Mr *S Kalvenes* of the Norsk Regnesentral and computed on the CDC 3300 at the Department of Electronic Data Processing at the University of Oslo

For cophenetic correlation analysis (40), the cophenetic values have been the per cent similarity level in the phenogram concerned at which two corresponding ONU's fuse Correlation analysis has been performed according to *Weber* (46)

RESULTS

Characteristics of the Strains of Pseudomonas aeruginosa

The variety of phage susceptibility among the bacterial strains used has considerable bearing on the validity of the comparison of the bacteriophages In all from abroad came 199 of the strains, 87 of which were the propagating strains for the phages, 284 were collected over a three year period from

* 'Operational numerical unit (ONU) is presently employed to denote the elements to be grouped corresponding to the term 'operational taxonomic unit' used in numerical taxonomy (43)

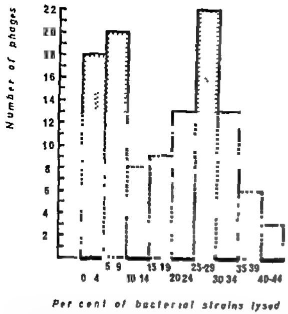


Fig 1 Variability of phage susceptibility for the 486 strains of *Pseudomonas aeruginosa* tested with 113 different typing phages at Routine Test Dilution (RTD) The histogram shows the number of phages lysing each of the portions (per cent) of bacteria indicated

specimens at this hospital (Table 2) One aspect of the variability of phage susceptibility is illustrated in Fig 1 That there are both highly susceptible bacterial strains and strains which only react with selected phages indicates a good and relatively varied selection of pseudomonas strains

Frequency Distribution of the Coefficients of Association

The range of the similarity coefficients is indicative of the diversity within the 113 phages The frequency distributions of the S_{JS} and S_{SM} values are shown in Figs 2 and 3 It is apparent that the mode value for the S_{JS}-matrix is considerably lower than for the S_{SM}-matrix The mean S_{JS} is 13 05 and for S_{SM} 73 36

Phenetic Classification of the Phages According to Their Host Ranges

a Comparison of the Jaccard Sneath similarity index and the simple matching coefficient The phenograms for the WPGA and the UWPGA based on [S_{JS}] (the S_{JS} matrix) and the UWPGA for [S_{SM}] are shown in Figs 4–6 The greater ranges of cophenetic values for the [S_{JS}] sorting procedures reflect the lower value mode for S_{JS} It is seen that e g strains 5 and 6 which are joined at a relatively high similarity level, in all phenograms with the S_{JS} formula have an 88 per cent similarity, whereas the similarity with the S_{SM} expression is 95 per cent The circumstance that the sorting strategies based on the [S_{JS}] yield cophenetic values which numerically are lower than in the corresponding procedures based on [S_{SM}] is of little importance, the main concern being the position of the ONU's relative to each other

Based on the experience with numerical taxonomy where several workers found S_{SM} to be preferable to S_{JS} at the outset it was intended to use only S_{SM} for the numerical allocation of bacteriophage lytic spectra However, the phenograms showed that bacteriophages lysing less than 15 per cent of the pseudomonas strains, tended to join the same cluster at a high level of similarity This occurred even when mostly different bacterial strains were lysed so that, intuitively, the phages would be considered widely different This could have been predicted from the formula for S_{SM} in that an ONU pair achieves a high S_{SM} similarity when the number of negative matches are overwhelming even when the relatively few positive reactions occur with entirely different bacterial strains This circumstance is illustrated in Fig 7 where the maximum S_{SM} value obtained for a phage is plotted against the percentage of bacterial strains lysed by it It turned out that phages with less than 15 per cent lysis, showed a strong correlation between the percentage of lysis and per cent similarity, whereas more avid phages are more randomly distributed in the scattergram This mechanism underlies the 99 per cent level clustering of ONU's 19, 50, and 53 with S_{SM} as a basis The host ranges of these 3 phages were entirely different *In this in*vestigation, probably partly due to the large

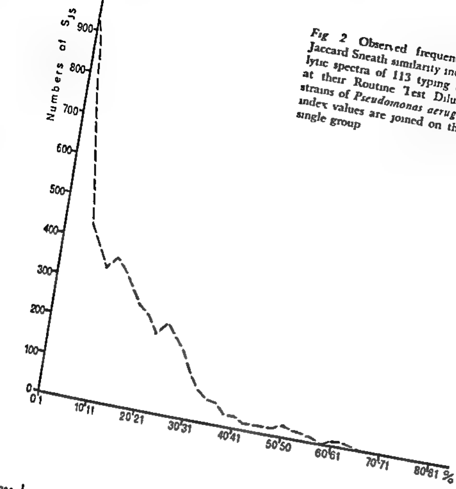


Fig 2 Observed frequency distribution of the Jaccard Sneath similarity index (S_{JS}) based on the lytic spectra of 113 typing bacteriophages applied at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa* Two similarity index values are joined on the x axis to form one single group

Fig 3 Observed frequency distribution of the simple matching coefficient (S_{SM}) based on the lytic spectra of 113 typing phages applied at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa* Two similarity index values are joined on the x axis to form one single group

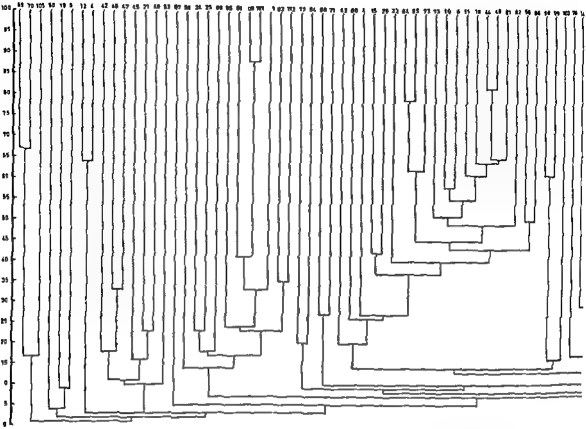


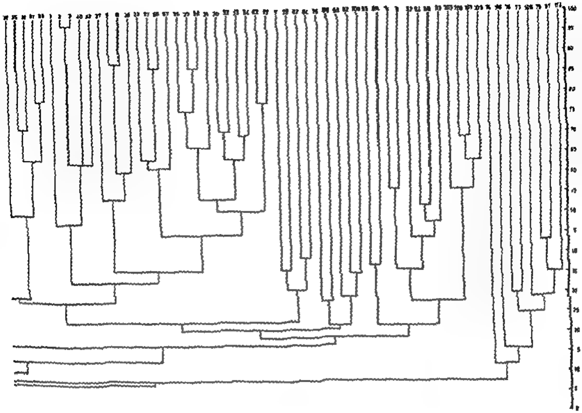
Fig 4 Phenogram achieved by the weighted pair group average cluster analysis (WPGA) of the lytic *aeruginosa* based on the Jaccard Sneath similarity index (S_{JS}) The abscissa is divided in per cent simi

ONU *vs* character matrix, the dendrograms based on S_{SM} showed pronounced chaining Fairly good separation was reached only below the 75 per cent phenon line, where several clusters had formed and four single ONU's (14, 52, 78, 93) remained unclustered The distinction achieved in the [S_{SM}] WPGA was even less

After these findings, it was decided to investigate clustering analysis also on the basis of the S_{JS} index The relationships among the above mentioned phages 19, 50, and 53 are more realistically portrayed by the S_{JS} dendrograms (Fig 4–5) which show ONU 19 to join ONU 50 at the 3 per cent level and the ONU 53 to join the others only at the 2 per cent level in WPGA and at the 3 per cent level in the UWPGA

Since S_{JS} only includes positive matches in the numerator, the question was whether any relationship was found between the percentage of bacterial lysis and the similarity index Fig 8 demonstrates that phages with a higher percentage of bacterial lysis also tend to have higher maximum S_{JS}'s This interdependence on the percentage of bacteria lysed and the maximum similarity index, however, is much less marked for S_{JS} than that which resulted from S_{SM} with the phages eliciting less than 15 per cent lysis It appeared that chaining was considerably less accentuated in the S_{JS} dendrograms than after employment of S_{SM} The longer more distinctive intervals between bifurcations appeared only in the upper level of the S_{JS} phenograms

As could be expected after these findings, a low correlation was found between the [S_{JS}] and the S[SM] ($r = -0\,1254$), a circumstance which is also illustrated in Fig 9 for the first fusion values of all *ONU s for* each index in the UWPGA



spectra for 113 bacteriophages at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas* larvy

b Comparison of the weighted and the unweighted pair group cluster analysis By simple inspection of the phenograms, it was apparent that there were similar cluster configurations in the WPGA and the UWPGA for each similarity index but that their dendrogram positions differed somewhat Cophenetic analysis confirmed these impressions (Table 3) The correlation coefficient between the WPGA and the UWPGA for [Sjs] was 0 9313 and for [Ssm] 0 6684 This relates primarily to the precision in the sorting procedures, not to the way in which each similarity matrix represents the original data matrix The Figs 10 and 11 demonstrate the two way frequency distribution of cophenetic values for each of the coefficients of association employed

In both Sjs dendrograms, the following clusters were quite similar in ONU patterns 59-105, 50-51 42-49 37-38, 22-67, 26-72, 7-84, 75-104, 8-109 In 96-112, the ONU's 24, 25 and 89 showed slightly different affinities The [Sjs] WPGA 80-86 complex and the [Sjs] UWPGA 4-86 complex appeared to have different affinity to the group 98-102 The [Sjs] UWPGA 14-68 complex was split in two between ONU 66 and ONU 3 in the WPGA procedure, and although grouped as adjoining clusters, are differently related to other ONU's The 110-113 complex in [Sjs] UWPGA and 74-113 in [Sjs] WPGA had different intragroup arrangements Whereas these clusters showed somewhat different patterns in the two sorting strategies, there were only small differences in cophenetic values within clusters and these could be insignificant with the low levels of similarity in question Several single or double items were placed differently in the two dendrograms e g 43 73 17-94, 55-85, and 60-71

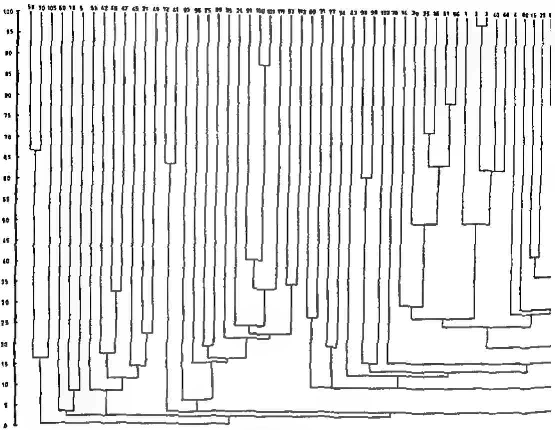


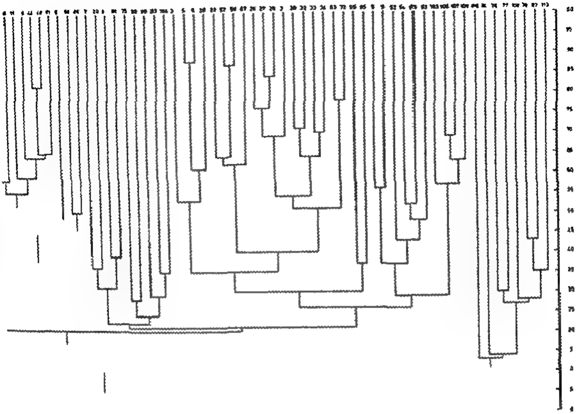
Fig 5 Phenogram achieved by the unweighted pair group average cluster analysis (UWPGA) of the *aeruginosa* based on the Jaccard Sneath similarity index (S_{JS}) Abscissa as in Fig 4

When the [S_{SM}] was processed, the following clusters became fairly similar in the two sorting procedures 8–85, 37–38, 1–68, 103–109, 26–72, 39–66, 16–86 with minor rearrangements, and roughly also the 17–113 complex The intragroup arrangements between these differed with the two procedures A few ONU's did not display affinity to any particular cluster, *e g* ONU s 14, 52, 78, and 93

An essential question at this point is which phenogram produces the least distortion of

TABLE 3 *Correlation between the* Jaccard Sneath *(S_{JS}) and the Simple Matching (S_{SM}) Coefficients of Association and the Cophenetic Values of the Weighted and the Unweighted Pair Group Average Cluster Analysis (WPGA and UWPGA) Based on the Host Ranges of 113 Bacteriophages at Their Routine Test Dilution (RTD) on 486 Strains of* Pseudomonas aeruginosa

	S_{JS}	S_{SM}	[S_{JS}] WPGA	[S_{JS}] UWPGA	[S_{SM}] WPGA
S_{JS}	—				
S_{SM}	—0 1254	—			
[S_{JS}] WPGA	0 8933	—0 1144	—		
[S_{JS}] UWPGA	0 9303	—0 1671	0 9313	—	
[S_{SM}] WPGA	—0 0100	0 6703	0 0310	—0 0150	—
[S_{SM}] UWPGA	—0 1763	0 9073	—0 1214	—0 1617	0 6684



for 113 bacteriophages at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas*

the data matrix There was no direct test available for this but the problem of the least distortion of similarity matrices has been analysed by covariance analysis Both sorting procedures have higher correlations to the [S_{JS}] As seen in Table 3 the UWPGA has the highest correlation coefficient with each of the similarity indices Consequently the [S_{JS}] UWPGA produces the most faithful representation of the similarity matrix with r=0 9303

DISCUSSION

Numerical allocating procedures seem to represent the most efficient tool conceivable for evaluating the lytic spectra of typing bacteriophages These procedures are objective and modern electronic computers allow a precise evaluation of large bodies of data However, these procedures presently must still be regarded as largely experimental such that it is important first to check the procedures applicability to the problem at hand Methods which have been valuable tools in taxonomy, might not perform as well within the framework of another sort of problem Before applying the procedures consequently it was desirable to examine their strong points and possible pitfalls Also it was not known how the procedures would manage a data matrix of the present size since any study approaching the present 54 918 entries has been unknown The results presented stress the paramount importance of a preceding methodological study

Certainly the value of the conclusions drawn from the comparative study of bacteriophage lytic spectra is related to the overall properties of the bacterial strains on which

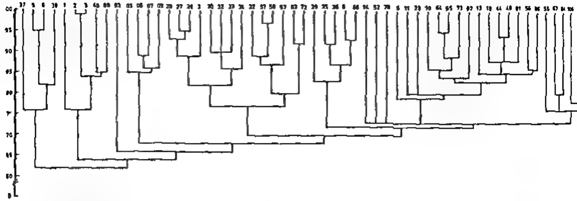


Fig 6 Phenogram obtained by the unweighted pair group average cluster analysis (UWPGA) of th nas aeruginosa based on the simple matching coefficient (S_{SM}) Abscissa as in Fig 4

the phages have been tested In this study, the number of attributes (bacteria) has been high The number of characters that would be necessary for a problem would depend on the particular grouping problem at hand *Sokal and Sneath* (43) indicate that 60 characters were an appropriate number for taxonomic studies, but certainly a higher number is necessary to ascertain a reasonable variation in phage receptor constellations for the present study The largest possible spectrum of pseudomonas strains has been sought by (i) including 199 strains from various sources abroad (Table 2) and (ii) collecting 284 domestic hospital strains over as long a period as three years and from a variety of sources within the entire hospital Since the typing set has been intended for human

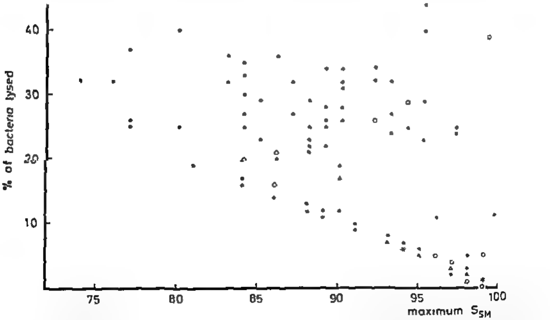
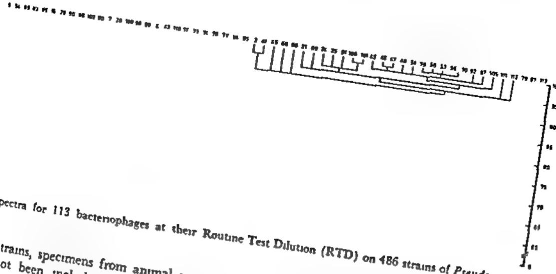


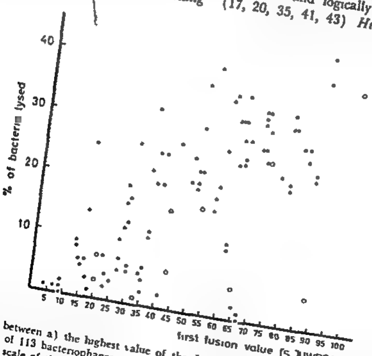
Fig 7 Relationship between a) the highest value of the simple matching coefficient (S_{SM}) for the lytic spectra of each of 113 bacteriophages and b) by the percentage lysed among 486 strains of *Pseudomonas aeruginosa* The scale of the abscissa indicates per cent similarity Explanation of graph symbols • = 1 observation ◘ = 2 observations, Δ = 3 observations * = 5 observations



c spectra for 113 bacteriophages at their Routine Test Dilution (RTD) on 486 strains of *Pseudomo*

strains, specimens from animal sources have not been included The variability in the percentage of phage susceptibility appears in Fig 1

The contribution of the comparative methodological study is illustrated by the fact that without it the simple matching coefficient (S_{SM}) would actually have been the one chosen From the results and reasoning of others, the S_{SM} appeared reliable and preferable to alternative coefficients of association In Adansonian numerical taxonomy, 'matches for 'negative' and 'positive' states" are of equal value and interest (43) The inclusion of all negative and positive characters has repeatedly been considered a reasonable and logically defensible position (17, 20, 35, 41, 43) *Hill et al* (13) con-



ig 8 Relationship between a) the highest value of the Jaccard Sneath similarity index (S_{JS}) for the tic spectra of each of 113 bacteriophages and b) the percentage lysed among 486 strains of *Pseudomo as aeruginosa* The scale of the abscissa and the symbols of the graph are explained Fig 7 The correla on coefficient between the two variables is 0 6725 the level of significance $\alpha < 0.001$ for a positive cor lation

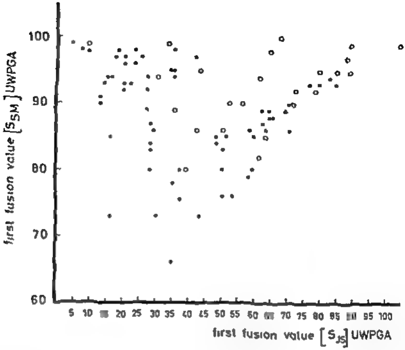


Fig 9 Relationship between the first fusion value for each bacteriophage based on the phenograms in Figs 5 and 6 The scale of the abscissa and ordinate indicate per cent similarity The symbols of the graph are explained Fig 7

sidered S_{SM} to yield more useful results The S_{SM} was found to be independent of the number of responses to which the + sign had been attributed The converse was found for the S_{JS} (8) *Hill et al* (13) consequently felt that the ratio of negative to positive reactions should be close to 1, if the S_{SM} were used By incorporating positive as well as negative matches, account is taken for the fact that positive *and* negative reactions both may indicate *presence* of genetic information Accordingly, it was rather surprising to find that the S_{SM} furnished completely confusing affinity coefficients The consequences of including negative matches depend on the ONU population at hand It is interesting to note that similar results were obtained by *Moffett & Colwell* (30) with data for the *Rhizobiaceae* In taxonomy, scoring the absence of single characters such as production of shigella toxin would be totally absurd when comparing such distant taxons as shigella, proteus, klebsiella, pseudomonas and sphaerophorus Emphasis could reasonably be put on positive matches when old broches were to be classified (14) In formulating a classification of aromatic substances according to their power to excite the glomeruli of the *bulbus olfactorius* (7), it was meaningful to consider all matches, negative and positive

Presence or absence of bacterial lysis should conceivably furnish equally important information regarding the qualities of the bacterial phage receptors In typing, however, the positive reactions are the focus of interest This relates well with the finding that S_{JS} better represented the phage host spectra *Sneath* (39) excluded negative matches in calculating his index because in taxonomy, he regarded the sphere of negative traits as essentially infinite and was unable to decide which negative features to exclude For instance, the 'absence of feather' was not applicable when comparing bacteria, whereas this character could be used in comparing bacteria and birds (43) *Sokal & Sneath* (43) reasoned that through *reductio ad ab*

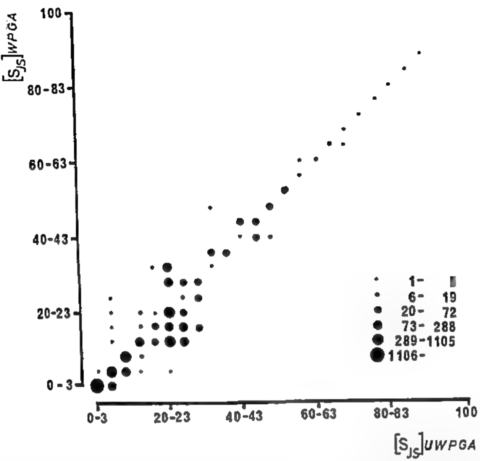


Fig 10 Two way frequency distribution of cophenetic values for the unweighted and weighted pair group average cluster analysis (UWPGA and WPGA) based on the Jaccard Sneath similarity index (S_{JS}) for the lytic spectra of 113 bacteriophages at Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa* The abscissa and ordinate indicate the cophenetic values in per cent Each cell in the graph has been obtained by uniting groups of four per-cent values on each axis The magnitude of the frequency at each point is indicated by dots of diameter varying according to the key in the lower right hand corner The correlation coefficient between the two variables is $r = 0.9313$

surdum one could arrive at a universe of negative trait matches which purported the establishment of affinity between any two entities This situation applied to that which occurred in the present instance with bacteriophage host ranges when S_{SM} was calculated for pairs demonstrating an overwhelming majority of negative reactions By implication the opposite argument would hold if there were mostly positive reactions, when the negative features would be the differentiating ones Solely positive matches have been included also in a larger series of other coefficients of association (6, 10, 22, 26, 32, 44)

The choice of an appropriate sorting procedure does not constitute quite the same dilemma The factor analysis procedures are more mathematically involved and do not produce the same kind of hierarchical like distinction as the cluster analysis Among the clustering techniques the average linkage procedures of *Sokal & Michener* (41) appear superior (23 39, 43) The average cluster

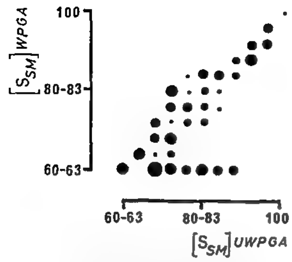


Fig 11 Two way frequency distribution of cophenetic values for the unweighted and the weighted pair group average cluster analysis (UWPGA and WPGA) based on the simple matching coefficient (S_{SM}) Explanations of scales and magnitude of the graph elements are as indicated Fig 10 The correlation coefficient between the two variables is r = 0 6684

analysis exhibited approximately the same affinities as factor analysis (31, 36) However, there are four alternative average clustering strategies

The provisions for admitting items to a cluster may be designed such that principally dichotomies evolve, the pair group method, or such that several items which lower the similarity by less than a chosen decrement are admitted per clustering cycle, the variable group procedure The arbitrary choice of a decrement value, however, is open to criticism The pair group method causes the least distortion of the similarity matrix since it detects even small differences in affinity (43) But, the pair group technique may force division of overlapping items that would tend to belong together The centroid of a cluster may be too distant from an ONU under consideration for it to be entered during a given cycle, even when the ONU has its most related individual within the cluster In the end, such an ONU is apt to join a cluster where there might be little relationship to any member already included (18) The variable group method produces fewer clusters The pair group method may result in mean similarity ($\bar{S}_n$) decrements that are too small to be graphically expressed In the presently used sorting program, the S_n's have been written without decimal places The pair group method correlates better with the evolutionary change of one phage host spectrum into another, *e g* by host modification One argument in favour of the variable group method is that it requires less computer time The merits of the (weighted) variable group cluster analysis in relation to classical concepts of taxonomy have been investigated previously (29) *Sokal* & *Rohlf* (42) compared four procedures and found that pair group average clustering produced the highest cophenetic correlation *Irimey Cook* (18) in summing up his experiences found that the pair group clustering was preferable to a variable group method This is also in agreement with the above

Somewhat more difficult to evaluate in advance was the effect of different procedures for calculating the average cluster similarity The cluster stems may be weighted equally the weighted method (WPGA) or each of the ONU s may contribute equally the unweighted method (UWPGA) The procedures have been explained in detail by *Sokal* & *Michener* (41) and *Sokal* & *Sneath* (43)

The weighted method was considered theoretically less objectionable by *Sokal* & *Sneath* (43) who preferred it until functions of intermediate weighting had been developed At the time, the relative merits of the two methods were basically unresolved although they (43) stated that there could be legitimate reasons for preferring the unweighted procedure in certain studies *Sokal* & *Michener* (41) found minimal difference between weighted and unweighted methods in a taxonomic study *Kendrick* & *Proctor* (21) and *Gower* (10) favoured the weighted procedures

For application to our problem of differentiating phage host ranges, the weighted procedure seemed less desirable since therein the contribution of each ONU decreases proportionately by increments in cluster size

With 113 ONU's, one might expect clusters to become large The weighted method might also entail lower end cluster homogeneity, since the last ONU s admitted have a stronger impact on cluster compositions These considerations were verified by the finding that the UWPGA had the highest cophenetic correlation with the two similarity matrices employed $r = 0.9303$ for [S_Js] and $r = 0.9073$ for [S_{SM}], which is in accordance with the experience of *Boyce* (4) and of *Sneath* (39) By inspection of the dendrograms, one may confirm the fairly similar results obtained for the two sorting procedures with each similarity index, whereas only small similarities may be identified for the two matrices relative to each other

In the cophenetic correlation analyses performed, the cophenetic value was defined as the fusion levels for each ONU pair This resulted in more accurate calculations than the alternative method (42) where the niveau of fusion was estimated by dividing the phenogram into a small number of classes

The findings of a relatively lesser difference in correlations between WPGA and UWPGA for each similarity matrix and greater differences in correlation between matrices are ascribable to properties inherent in the cluster analysis procedures as such

Since the S_{SM} rendered a linear relationship between the per cent of the bacterial strains lysed and the maximum similarity index value for each phage, and the cluster analysis based thereon contained considerable chaining the S_Js phenograms were preferred for the further evaluation of the relationship between the bacteriophage host ranges (1) The most favourable similarity matrix representation was rendered by the [S_Js] UWPGA (Table 3)

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EVALUATION OF PHENOLICS BY A QUANTITATIVE TECHNIQUE

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The quantitative technique of the British Standard (BS 3286 1960) has been applied to four phenolic disinfectants Comparisons showed that regressions made with log probit transformations were preferable to log log The British Standard quantitative technique as employed by us has been found to act as a useful adjuvant to *Kelsey & Sykes* (1969) capacity test in the selection of use dilutions for disinfectants

A previous report has presented the use of a quantitative procedure described by the British Standard (BS) number 3286 1960 for the evaluation of quaternary ammonium compounds (QAC's) as applied to various non phenolics Presently, the technique has been applied to four phenolics These results will be presented and the technique evaluated on the basis of this and a previous communication (*Bergan & Lystad* 1972)

MATERIALS AND METHODS

The test procedure has been described in detail previously (*Bergan & Lystad* 1972) The specific inactivators were adapted from Bergan & Lystad (1971)

Disinfectants

1 Arylated and alkylated phenolics (Ivisol®, Schulke & Mayr, GMBH) (Disinfectant D)
2 p-Chlor m-cresole (16 3 per cent) (Talosan®, William Nagel A/S) (Disinfectant F)
3 o-Benzyl p-chlorophenol (5 2 per cent w/w) and o phenylphenol (1 0 per cent w/w) (Vesphene Instrument®, A/S Kistner, batch no 278 29 JV) (Disinfectant G)
4 o Phenylphenol (45 per cent) (Fenylfenolat såpe®, Norsk Medisinaldepot (Disinfectant H)

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RESULTS

Comparison of Statistical Procedures

The results are presented in Tables 1–8 With disinfectant D, the effective dose (ED) obtained by the log-probit transformation is within the range suggested by disinfectant concentrations straddling the end points for all strains except strain no 8 Here, the ED is slightly high, but certainly within the range found in other *Escherichia coli* strains With the log-log transformation two strains (1 and 2) received slightly higher ED s than expected, and three results (strains 17, 23, and 24) were too low For two of the latter, the goodness of fit was unsatisfactory for both transformations

The results with disinfectant D could be better represented by log probit transformation Whereas most of the ten determinations have resulted in reasonable ED s in

TABLES 1-4 *The Mean 99 99 Per Cent Kill Concentration (ED) of Each Disinfectant against Bacterial Pathogens with the Unweighted Least Squares Regression Slope (b) and Their Standard Errors (SE(ED) and SE(b)) and the Regression Goodness of Fit as Determined in Quantitative Assays The Disinfectant Concentrations and Per Cent Kill Were Both Logarithmically Transformed*

	Strain	Goodness of fit $P(\chi^2 \geq \alpha) =$	Regression coefficient (b)	SE(b)	Effective dose (ED)	SE(ED)
Table 1 Disinfectant D	1	0 99	0 5	3 31	0 39	0 98
	2	0 99	1 1	2 32	0 42	0 40
	3	0 975	0 1	4 68	0 53	7 95
	7	0 99	1 1	1 34	0 40	0 30
	8	0 975	0 1	4 68	0 52	6 2
	11	0 995	0 5	3 97	0 34	0 97
	13	0 99	0 7	2 32	0 42	0 60
	14	0 99	1 3	2 47	0 37	0 39
	17	0 01*	15 4	4 68	0 27	0 03
	19	0 9	1 5	4 07	0 39	0 28
	20	0 99	1 8	3 85	0 47	0 30
	23	0 995	0 7	2 77	0 69	1 32
	24	0 001*	25 1	3 22	0 59	0 039
	25	0 95	1 7	2 93	0 58	0 40
	28	0 0001*	29 4	5 4	0 82	0 051
	34	0 95	0 9	1 49	1 28	2 05
Table 2 Disinfectant F	1	0 3	0 8	2 35	2 92	16 61
	2	0 995	0 2	1 66	1 64	18 78
	3	0 99	0 6	1 18	1 76	3 57
	7	0 9	1 0	2 35	1 04	2 24
	11	0 025*	5 2	1 49	1 34	0 38
	13	0 975	2 3	2 00	1 14	0 83
	14	0 05	8 3	2 35	0 83	0 18
	19	0 995	0 4	1 05	1 92	7 44
	20	0 995	0 6	1 49	1 89	5 67
	22	0 995	0 01	1 40	1 30	262 76
	23	0 9	0 8	1 49	1 28	2 36
	24	0 1	8 3	2 35	0 87	0 20
	25	0 7	1 7	2 35	0 88	0 95
	28	0 99	0 5	1 22	2 16	6 41
	29	0 995	0 5	1 99	2 22	7 21
Table 3 Disinfectant G	2	0 995	1 0	1 10	1 31	1 36
	3	0 95	0 5	2 28	4 26	31 34
	7	0 95	7 0	8 07	1 35	0 33
	8	0 0001*	41 8	5 51	1 53	0 044
	11	0 99	1 5	1 27	1 34	0 80
	13	0 9	1 2	3 30	1 16	1 58
	14	0 1	6 6	2 35	0 81	0 21
	23	0 99	0 3	3 15	1 77	5 75
	24	0 99	0 7	1 79	1 19	2 95
	28	0 99	0 6	1 94	1 67	2 90

* Asterisks indicate that the goodness of fit for the regression is not significant ($P < 0\ 05$)

	Strain	Goodness of fit $P(\chi^2 \geq \alpha) =$	Regression coefficient (b)	SE(b)	Effective dose (ED)	SE(ED)
Table 4	1	0 7	3 4	3 20	0 31	0 14
Disinfectant H	7	0 99	0 5	0 85	0 40	0 81
	8	0 3	1 7	4 67	0 45	0 48
	11	0 95	1 8	10 1	0 33	0 42
	13	0 975	0 09	4 67	0 44	8 50
	17	0 9	1 4	4 58	0 62	0 77
	19	0.5	3 9	4 67	0 45	0 21
	20	0 995	5 0	4 07	0 25	0 12
	24	0 995	0 8	4 56	0 68	0 16
	25	0 99	0 3	1 56	1 49	7 24

TABLES 5–8 *The Mean 99 99 Per Cent Kill Concentration (ED) of Each Disinfectant against Bacterial Pathogens with the Unweighted Least Squares Regression Slope (b) and Their Standard Errors (SE(ED) and SE(b)) and the Regression Goodness of Fit as Determined in Quantitative Assays The Disinfectant Concentrations and the Per Cent Kill Have Been Transformed with Logarithms and Probits Respectively*

	Strain	Goodness of fit $P(\chi^2 \geq \alpha) =$	Regression coefficient (b)	SE(b)	Effective dose (ED)	SE(ED)
Table 5	1	0 2	12 5	3 31	0 36	0 035
Disinfectant D	2	0 3	10 1	2 32	0 41	0 038
	3	0 5	7 9	4 68	0 54	0 12
	7	0 3	9 5	1 34	0 38	0 030
	8	0 1	7 9	4 68	0 51	0 11
	11	0 6	9 5	3 97	0 39	0 081
	13	0 2	9 3	2 32	0 37	0 035
	14	0 005*	13 5	2 47	0 30	0 024
	17	0 001*	17 5	4 68	0 36	0 058
	19	0 8	14 9	4 07	0 44	0 041
	20	0 2	15 6	3 85	0 48	0 038
	23	0 8	7.3	2 77	0 94	0 26
	24	0 1	24 1	3 22	0 63	0 046
	25	0 9	9 7	2 93	0 79	0 15
	28	0 0005*	34 2	5 41	0 83	0 046
	34	0 8	7 8	1 49	1 20	0 21

* = Asterisks indicate that the goodness of fit for the regression is not significant ($P < 0\ 05$)

the log probit system, only five of the log-log calculations were consistent with the ranges suggested from the concentrations straddling the end points Strain 25 gave absurdly high ED s in both coordinate nets, but the log-probit goodness of fit to the line was P < 0 001 Poor linearity of the determinants resulted with strain no [illegible] in the log-log and strain no 3 in the log-probit system

With disinfectant F, the log-probit regression led to too high values in five out of fifteen instances The log log regression led to four ED s being too low, two ED s being too high, and one having an unsatisfactory goodness of fit

With disinfectant G the log log regressions rendered low ED s in all 10 cases except for strains nos 3 7 13 whereas the log probit transformation rendered satisfactory ED's in all but strains nos 14 and 23 where the ED's

	Strain	Goodness of fit $P(\chi^2 \geq \alpha) =$	Regression coefficient (b)	SE(b)	Effective dose (ED)	SE(ED)
Table 6	1	0.05	3.3	2.35	7.24	13.89
Disinfectant F	2	0.3	4.6	1.66	1.43	0.61
	3	0.2	5.9	1.18	1.86	0.42
	7	0.9	3.1	2.35	6.77	13.52
	11	0.8	9.5	1.49	1.66	0.30
	13	0.3	6.6	2.00	2.17	0.92
	14	0.8	12.1	2.35	1.12	0.21
	19	0.4	3.5	1.05	3.45	2.19
	20	0.3	4.6	1.49	2.87	1.48
	22	0.5	1.8	1.4	1.39	1.98
	23	0.5	5.3	1.49	1.60	0.50
	24	0.4	12.8	2.35	1.11	0.19
	25	0.6	7.0	1.46	1.51	0.34
	28	0.1	5.0	1.22	2.33	0.71
	29	0.4	6.7	1.99	2.41	0.64
Table 7	2	0.1	4.0	1.10	3.35	1.60
Disinfectant G	3	0.025*	5.9	2.28	3.17	1.46
	7	0.5	22.4	8.07	1.65	0.23
	8	0.2	43.7	5.51	1.62	0.051
	11	0.8	7.1	1.27	1.61	0.23
	13	0.05	11.9	3.30	1.08	0.14
	14	0.6	12.2	2.35	0.98	0.17
	23	0.975	9.6	3.15	1.96	0.26
	24	0.2	5.6	1.79	1.61	0.58
	28	0.7	7.2	1.94	1.97	0.32
Table 8	1	0.2	10.3	3.21	0.46	0.12
Disinfectant H	7	0.3	3.8	0.85	0.75	0.29
	8	0.7	16.5	4.67	0.44	0.047
	11	0.8	18.8	10.16	0.38	0.073
	13	0.3	7.9	4.67	0.41	0.083
	17	0.5	12.8	4.57	0.71	0.12
	19	0.7	17.2	4.67	0.50	0.063
	20	0.995	17.6	4.07	0.26	0.036
	24	0.2	10.9	4.56	0.77	0.17
	25	0.9	4.7	1.56	2.10	0.90

* – Asterisks indicate a not significant goodness of fit for the regression ($P < 0.05$)

were slightly lower than expected. Each transformation had one case where the goodness of fit was not significant.

For disinfectant H all strains exhibited a significant goodness of fit. With the log probit axes two ED's were somewhat high (strains 8 and 25) and one low (strain no. 20) as opposed to two low (strains nos. 1 and 11) and one high (strain no. 13) determinations with the log-log transformations. As determined by only two observations in the interval below 99.99 per cent kill, the mean ED's for the *E. coli* strains 2 and 3 were 0.5 and 0.67 per cent respectively* with the log log transformation and 0.49 and 0.73 per cent with the log probit axes.

* All disinfectant concentrations throughout this paper indicate percentages of the undiluted commercial product (*as is*).

Experimental Variation

The variation in the determinations is discernible from the standard errors (SE) to the ED and slope (b) and by the goodness of fit as evidenced by the χ^2 values calculated for the determinates upon their regressions as reported in Tables 1–8 With identical experiments performed on different days some differences in ED s were observed

DISCUSSION

Variation

Quantitative techniques for disinfectant evaluation are quite exacting The viable counts of bacteria follow a Poisson distribution (*Berry & Michaels* 1950) but replicate counts of cells exposed to disinfectants vary more than would be expected particularly with mortalities greater than 95 per cent (*Jordan & Jacobs* 1944ab *Withell* 1942), even if continuous cultures are employed These conclusions were made with phenol in experiments where reduction of the antibacterial effect upon subculture was achieved entirely through dilution not by specific inactivators The variation may be reduced some by gentle subculture methods and selection of a rich culture medium or addition of charcoal to the subculture medium (*Sykes* 1962 1965) Another abberration to be considered in estimating survival is the variations in resistance within a population which has been observed for both vegetative forms and spores (*Kronig & Paul* 1897 *Jacobs* 1960)

Curves

Calculations of the disinfectant concentrations rendering 99 99 per cent kill (ED) are based on assumptions of rectilinearity in log probit or log log nets Both for phenolics and for the previously reported non phenolics (*Bergan & Lystad* 1972) the log probit regressions were more satisfactory than those in log log since they rendered significant goodness of fit for the regression in more instances and the ED s correspond better to the range defined by data straddling the end point

The preference for probit plots is shared by others *Cook & Wills* (1954) found log time probit kill regressions to be statistically significant over a larger portion of the curves Log probit rectilinearity is most pronounced in the range of 4–6 probit units (*Berry & Michaels* 1950) and has been used for disinfectant potency controls *Ortenzio & Stuart* (1961) used probit plots for cylinders without growth in the use dilution test of the Association of Official Agricultural Chemists (AOAC) to determine the log concentrations eliminating growth in 99 per cent of the cylinders Dealing with less than 95 per cent kill his results probably are not fully valid for inactivation factors of the size presently used but *Withell* (1942) found normal distribution of log time for a micrococcus and an *E coli* strain using 0 5 per cent phenol and 0 05 per cent p chlor m cresol such that deviations from rectilinearity in probit nets were insignificant although *E coli* occasionally displayed an inflexion around 4 5 probit units of kill

Nevertheless log log transformations as has been suggested by the BS 3286 1960 would also be acceptable in a number of instances for our data Log log dose response curves have previously showed variations in shape (*Withell* 1942) and linearity observed for virtual sterilization time against log phenol concentrations for *E coli* (*Jordan & Jacobs* 1946) Survival curves are probably only exponential when bacterial inactivation is sufficiently high (*Sykes* 1965) In fact when a certain regression procedure is adopted it is because it facilitates calculation although with the assumption that adherence to the model is close enough to render useful results However due to estimate errors the exact shape of the curves cannot be predicted throughout their entire course Borrowed arguments from the hit theory of quantitative radiation biology (*Zimmer 1961*) will obviate this Molecules of a disinfectant being microphysically heterogeneous the absorption into the cells and inactivation of vital metabolic processes (hits) are stochastic and therefore the Poisson law of distribution applicable

$$p_n = \frac{(VD)^n e^{-VD}}{n!}$$

where p_n = the probability of n hits in a volume V and D is the dose measured in hits per cm³ (*Zimmer* 1961) The number of cells (N) among a starting number N_o exposed which have not been inactivated after a dose D is $N = N_o e^{-VD}$ Accordingly, probability plots with logarithmic abscissas will be consistent with rectilinearity for curves describing 4 or more hits (*Zimmer* 1961) In addition, several different multicomponent curves may be drawn on the basis of one set of determinates By inference warnings have been made against claiming single-hit or multi hit kinetics for inactivation processes of this kind based on experimental determinations Particularly with higher inactivations than 10^4, deviations from a seemingly rectilinear plot may be considerable Although the above statistical considerations explain this phenomenon partly, there are decreasing death rates towards the end of the disinfection period with low disinfectant concentrations (*Sykes* 1965 *Jacobs* 1960 *Smith* 1921 *Withell* 1942) which may be explained either as a skew distribution of cell resistance or as disinfectant inactivation due to release of intracellular material (*Bean & Walters 1961*) Consequently a valid statistical evaluation of the BS quantitative technique requires that concentrations straddle the end point and are reasonably spread over a large portion of the logarithmic scale In the range below the 10^4 inactivation there must be at least three determinations preferably 7 or more to yield 5 or more degrees of freedom for the χ^2 estimation of goodness of fit The unavoidable day to day variation makes repetitions necessary

Regression Coefficients

As could be expected the mean regression slopes are dissimilar both for one strain with different disinfectants and for different strains with the same disinfectant, for phenolics and for the previously reported non phenolics (*Bergan & Lystad* 1972), although the standard errors of the slopes indicate that care is required in these evaluations However, the findings are supported by *Berry* and *Michaels*'s (1950) observations of differences in regression slopes for different organisms against the same disinfectant, and for systems that differed only in reaction temperatures

Strain Resistance and Methodology of the Quantitative Technique Employed

Basing our conclusions on the log probit ED s, it appeared that there was a tendency for a strain with a higher resistance against one disinfectant to exhibit a higher resistance also against another brand Although not a pronounced tendency for *E coli* it was distinct for klebsiella proteus pseudomonas and the staphylococci However, it was not possible to select any single strain among these which had a universal resistance against all disinfectants such that disinfectant evaluations must always be made with several strains However, if two of the indicators only had been used strains 11 (*P mirabilis*) and 20 (*P aeruginosa*), relatively high end point concentrations would have resulted with all non phenolics (*Bergan & Lystad* 1971b) or phenolics except for brands D G and H With brand D, *C albicans* was the most resistant with staphylococci as surprising seconds Strains nos 2 and 3 (*E coli*) were the most resistant against disinfectant G and strain no 25 (*S aureus*) against disinfectant H

These findings of the variability in resistance found with different strains conflict somewhat with the employment of only one test strain as is done in some procedures e g the Rideal Walker and Chick Martin phenol coefficient tests (BS 541 1934 BS 808 1938) which employ a salmonella strain Realising the variation in bacterial resistance the Kelsey test (*Kelsey & Sykes* 1969) employed four strains although it is interesting to note that those strains (here designated as nos 1 14 19 and 25) were exceeded in their *resist*

ance by strains selected from pathological material The AOAC phenol coefficient method and use dilution method (*Bass & Stuart* 1968) employ *Salmonella* sp *P aeruginosa* and *S aureus* It would be worth while to supplement these further with relatively resistant strains particularly of *P mirabilis* and *C albicans* The Deutsche Gesellschaft fur Hygiene und Mikrobiologie test battery (*Adam et al*, 1969) is quite alert to the importance of including many species although we have no comparative data to indicate to us the resistance of these particular strains relative to our own

When one compares our previous (*Bergan & Lystad* 1972) and present findings in the British Standard quantitative technique with the results of the capacity test, it appears that the latter has a considerable built in margin of safety Still, it is important to note that there is a relatively equal resistance profile for each of the two procedures The strains which appear as the most resistant ones in the BS procedure, are also generally the most resistant strains in the capacity test This circumstance may serve as a useful indication of the validity of both tests

At present, certainly, no single universally adoptable method for disinfectant evaluation is conceivable Quite likely, there will always be some disagreement in the details of tests for disinfectant assessment, even when limited uses are kept in mind Thus similarly to the fate of virtually all other techniques regardless of their wide acceptance, it is entirely conceivable that the BS quantitative test design will be considered by others not to be fully satisfactory The merits of the present quantitative technique is its demonstration of the dynamic relationship between varying disinfectant dilutions and bacterial kill A disadvantage is that it requires considerable technical experience before reasonably reproducible assays can be made The assay must be carried out with meticulous attention to detail and may seem time consuming considering the data output In our hands it has been satisfactory when the reproducibility exhibited by other techniques is considered By employing organic matter, a short time of action, and pathogens that are important in the hospital environment, this procedure should constitute a sound basis as an aid in the selection of valid use dilutions for disinfectants

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EVALUATION OF NON-PHENOLICS BY A QUANTITATIVE TECHNIQUE

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The minimum performance concentration of five non phenolics have been tested by the British Standard Quantitative Technique which is a suspension procedure By least squares regression in log probit and log log graphs, the 99 99 per cent kill concentrations of the disinfectants have been calculated For non phenolics, the unweighted log probit least square regression has been found preferable to end point determinations

Biological assay will always be required to indicate the effect of disinfectants As pointed out by the Public Health Laboratory Service Committee on the Testing and Evaluation of Disinfectants (1965), no single procedure or set of procedures has been generally accepted The phenol coefficients of *e g* Rideal-Walker (RW) (British Standard (BS) 541 1934) and Chick Martin (CM) (BS 808 1938) have their disadvantages (i) the validity of the results decreases according to increasing dissimilarity from phenol due to unequal antibacterial spectra, or the different impact of changes in pH, temperature, dilution, organic matter, and inorganic salts To attain a test which is both theoretically sound and practicable, various test designs have been elaborated Certain use-dilution tests sponsored by professional organizations have received wide acceptance, *e g* methods of the Association of Official Agricultural Chemists (AOAC) (*Bass & Stuart* 1968) and the test battery of the Deutsche Gesellschaft der Hygiene und Mikrobiologie (DGHM) (*Adam et al* 1969) These, however, are tests with a complete kill end point, a principle which has theoretical disadvantages (*vide infra*)

A rational approach to disinfectant evaluation would be to asses the factor of inactivation as in standard procedure for sterilization by heat and irradiation For chemical disinfectants, a procedure adhering to this principle has been outlined on the band of quaternary ammonium compounds (QAC s) by the British Standard 3286 1960

The purpose of this communication is to adapt this test design and determine the con centration of various non phenolics rendering an inactivation factor of 10^4

MATERIALS AND METHODS

The following strains were employed

Strains nos 1 5 *Escherichia coli*
NCTC 8196 – 5277/68 – 5569/68 – 5791/68 – 2576/68

Strains nos 6–10 *Klebsiella aerogenes*
2574/68 – 2555/68 – 5247/68 – 5208/68 – 5388/68

Strains nos 11 13 *Proteus mirabilis*
2502/68 – 2548/68 5257/68

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Strain no 14 *Proteus vulgaris* NCTC 4635
Strains nos 16–20 *Pseudomonas aeruginosa* 4343/68 – 5194/68 – 5172/68 – NCTC 6749 – 4624/68
Strains nos 21–25 *Staphylococcus aureus* 5226/68 – 5238/68 – 5207/68 – 5570/68 – NCTC 4163
Strain no 26 *Streptococcus pyogenes* (Lancefield group A) 6190/68
Strains nos 28–29 *Enterococcus* (Lancefield group D) WBG – 2593/68
Strain no 34 *Candida albicans* 5208/68

The NCTC strains, obtained from Dr *J C Kelsey*, London, were used by *Kelsey et al* (1965) The handling of the strains has been as described previously (*Bergan & Lystad* 1971)

For testing purposes, we used aerated cultures (shaker) incubated at 37° C for 18 hours in Nutrient Broth No 2 (Oxoid), To achieve a more even cell distribution, the cultures were further shaken with glass beads for 5 minutes before photometrical standardization with 10^9 cells per ml broth for use in disinfectant assays

Yeast Preparation

A 2 per cent dry weight yeast preparation of pH 6 9–7 1 was prepared according to the BS 808 1938 for the CM phenol coefficient method

Disinfectants

The germicides tested were

1 Cetylpyridinium chloride (5 per cent) (Aktivex® containing etylenediaminetetracetate (EDTA), Weiders Farmasøytiske A/S) (Disinfectant A)
2 Chlorhexidine gluconate (1 5 per cent w/v) with cetrimide B P (15 0 per cent w/v) (Savlon®, ICI) (Disinfectant B)
3 Picloxydine digluconate (1 0 per cent w/w) + octylphenoxypolyethoxyethanol (11 0 per cent w/w) + benzalkonium chloride (12 0 per cent w/w) (Resiguard® Nicholas Laboratories Limited) (Disinfectant C)
4 Glutaraldehyde (2 per cent) (Cidex®, Johnson & Johnson) (Disinfectant E)
5 Tolylchloramidnatrium Ph Nord (chloramine T, 100 per cent) (Kloramin®, Norsk Medisinaldepot) (Disinfectant I)

Test Temperature

All assays were performed in a water bath of 20° C which was also the temperature of all reagents,

Inactivators

Specific disinfectant inactivators were the same as used before (*Bergan & Lystad* 1971)

Glassware

All glassware used was meticulously washed and rinsed

Test Procedure

Tenfold dilutions of disinfectant in sterile tap water were distributed to large test tubes, each with an aliquot of 5 ml To every tube 5 ml of a 1 + mixture of a bacterial suspension with 10^9 cells and the yeast suspension was added After periods of 5 minutes, 1 ml of the medication mixture was added to 9 ml broth containing a neutralizing agent After a brief shaking, aliquots for viable counts were taken from the neutralizing tube Quantitation was performed in triplicate with the surface spread method Subculture plates of Nutrient Broth No 2 (Oxoid) with 1 5 per cent Agar (Oxoid) were incubated at 37° C for 48 hours before counting

Disinfectants without specific inactivators were assayed by a membrane filter method Suitable aliquots of the reaction mixture were filtered (Millipore, 0 45 μ with hydrophobic edge) followed by three washings of 100 ml broth each Subsequently, the filters were transferred to nutrient agar plates and the resulting colonies counted For technical reasons, the reaction time could not be as sharply defined with this procedure

Test End Point and Statistical Procedures

An inactivation factor of 10^4 (99 99 per cent kill) was chosen as end point To this end, two different statistical methods were employed and compared

According to the recommendations of BS 3286 1960, the per cent survival and disinfectant concentrations should be transformed logarithmically and the least squares regression calculated (*Weber* 1964) The nature of the results obtained and the wide use of probits in dose-response curves led to the calculation also of the unweighted probit log regression (*Bliss* 1952) The unweighted probit regression procedure was preferred to the weighted one which is frequently used The former was considered more valid for this problem due to the large number of individuals determining each point, the points with less than 10^4 inactivation *all* have appproximately the same predictive power Starting with 10^9 cells, the situation in the present assay differs from dose response evaluations where 5–50 odd individuals are more frequently included in a group In such cases the weighted *probit* procedures are preferable The data for phenolics (*Bergan & Lystad* 1972), indeed demonstrate the superiority of the unweighted procedure *for* our type of problem The effective dose (ED) of a disinfectant rendering 99 99 per cent *kill, was* extrapolated on the regression line according to

$$ED = \bar{x} - \frac{\lambda - \bar{y}}{b} \qquad (1)$$

The $\bar{x}$ and $\bar{y}$ are the means of the observations independent and dependent variate

The λ is the numerical value on the probit or on the $\log_{10}$ scale corresponding to 99 99 per cent kill The ED standard error was calculated (Bliss 1952) regression slope (b) and its standard error (SE(b)) were estimated (Weber 1961 Bliss 1952) Goodness of fit for the regression was calculated by a χ^2 approximation and considered significant for $P < 0.05$

RESULTS

Comparison of Statistical Procedures

The BS 3286 1960 recommended log log probits for obtaining straight line relationships between number of survivors and disinfectant concentrations Out of 15 determinations for disinfectant A (Table 1), the results obtained in 3 were reasonable, i e with strains 8, 17, and 24 two were somewhat high (strains 25 and 28), and the rest too low The goodness of fit was acceptable for all but strain 7 That the mean effective concentrations were reasonable could be evaluated in all cases since the end points always were straddled by several determinations on both sides

For disinfectant C the results (Table 2) showed that three out of 7 determinations were reasonable, one slightly high and three were too low The goodness of fit was significant for all the regressions The ED for disinfectant E was estimated correctly for all the four strains with acceptable regression goodness of fit (Table 3) Disinfectant I (Table 4) had significant goodness of fit in all instances but too low end point concentrations in two (strains 19 and 20) and a slightly excessive ED in one strain (3)

Since a considerable number of obviously erroneous endpoint determinations were obtained by the log log transformations in spite of a determinate variation within acceptable limits probit transformations were investigated

In dose response curve determinations probit analysis is standard procedure This rendered a significant goodness of fit in all cases but one In disinfectant A, the estimate for the EDs as evaluated by the bacterial kill in disinfectant concentrations straddling the end point appeared too high in strains 1 20, 24, and 28 The results with brands C and E were without particular aspects The EDs for disinfectant I was high in strains 8 20 and 25 (determined as 0 36, 1 76, and 0 51 per cent* respectively according to Table 8)

The results of the unweighted log probit calculations appear in Tables 5–8 This probit procedure resulted in entirely reasonable end point concentrations with disinfectants A and E. With brand C the value for strain 23 was insignificantly raised and for disinfectant I the ED was moderately raised with strains 20 and 25

Accordingly, the mean effective dose was reasonable in all cases with the log probit procedure whereas with the log log transformation the goodness of fit was unacceptable in four cases Inspection of the log log and log probit plots revealed that the latter followed the determinates better at the higher levels of kill

Disinfectant Concentrations Rendering 99.99 Per Cent Kill

It is seen that the log log transformation frequently renders EDs below that reached by log probit and that the latter caused better linearity Consequently, the subsequent discussion refers to the log probit regressions The highest ED for brand A (cetylpyridinium chloride) was 13 98 ± 7 49 per cent with a *P mirabilis* The pseudomonads and strain number 3 (*E coli*) were also fairly resistant Also with disinfectant C *P mirabilis* and *P aeruginosa* were the most resistant species Chloramine T is less effective against pseudomonads than against other species the highest ED being 1 76 ± 1 21 per c t The number of successful experiments w l gl taraldehyde where a membrane wasl e technique was

* Disinfectant concentrat o s throughout this paper indicate percentages of the und luted commercial product as is

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TABLES 5-8 *The Mean 99 99 Per Cent Kill Concentration (ED) of Each Disinfectant against Bacterial Pathogens with the Unweighted Least Squares Regression Slope (b) and Their Standard Errors (SE(ED) and SE(b)) and the Regression Goodness of Fit as Determined in Quantitative Assays The Disinfectant Concentrations and the Per Cent Kill Have Been Transformed with Logarithms and Probits Respectively*

	Strain	Goodness of fit $P(\chi^2 \leqq \alpha) =$	Regression coefficient (b)	SE(b)	Effective dose (ED)	SE(ED)
Table 5	1	0 3	8 3	2 28	2 03	0 45
Disinfectant A	2	0 95	6 5	2 57	3 22	0 79
	3	0 975	4 1	1 53	5 31	1 39
	7	0 01*	20 5	2 87	2 64	0 21
	8	0 6	7 0	4 67	4 51	1 16
	11	0 95	5 3	2 28	13 98	7 49
	13	0 95	7 8	2 27	6 11	1 45
	14	0 975	5 8	1 78	3 58	1 13
	17	0 6	7 0	2 13	5 07	1 76
	19	0 6	11 8	4 67	3 50	0 82
	20	0 7	6 3	1 50	5 06	1 17
	23	0 9	6 3	2 87	4 12	1 78
	24	0 6	6 0	2 27	1 16	0 41
	25	0 3	7 0	1 76	1 55	0 28
	28	0 95	4 4	2 28	1 10	0 29
Table 6	3	0 6	7 3	1 99	0 73	0 11
Disinfectant C	8	0 1	8 0	2 35	0 9	0 22
	11	0 7	6 1	2 21	1 94	0 65
	20	0 9	4 0	2 28	1 79	0 73
	23	0 8	7 9	1 49	0 67	0 068
	28	0 8	6 3	2 27	0 57	0 11
	34	0 9	5 6	1 27	1 80	0 27
Table 7	3	0 9	7 0	2 35	5 20	1 30
Disinfectant E	20	0 95	0 7	0 67	19 00	25 00
	23	0 6	4,2	1 05	5 50	2 20
	28	0 995	1 7	1 10	52 00	39 00
Table 8	1	0 3	9 6	2 67	0 076	0 011
Disinfectant I	3	0 1	2 7	3 40	0 72	1 87
	8	0 6	4 2	2 35	0 36	0 14
	13	0 6	15 8	4 05	0 058	0 0076
	14	0 2	7 9	2 28	0 11	0 026
	19	0 95	3 8	1 48	1 29	1 09
	20	0 95	5 2	2.50	1 76	1 24
	23	0 2	8 5	2 14	0 17	0 028
	24	0 1	7 8	2 28	0 18	0 038
	25	0 4	3 6	1 24	0 51	0 36
	28	0 975	12 1	4 07	0 27	0 055

* = Asterisk signifies not significant goodness of fit for the regression ($P < 0\ 05$)

Test Procedure Variation

The assay variation with non phenolics was partly indicated by the standard error (SE) to the mean ED partly by the χ^2-fractile in the goodness of fit for the unweighted regression, and partly by the SE to the regression coefficient These are discerned from the Tables 5–8 A day to day variation was also observed

6*

DISCUSSION

A procedure similar to the British Standard quantitative technique has previously been used by *Davis* (1949) He, also, added 1 ml of his bacterial suspension to 9 ml of a disinfectant dilution After a set reaction time, 1 ml was transferred to 5 ml of a 1 per cent Lubrol W solution for disinfectant inactivation, and viable counts made Another test which in every technical detail resembles the British Standard quantitative technique was used by *Ostrander & Griffith* (1962) They sampled the medication tube after 5 minutes as we did, but also after 10 minutes

The present quantitative procedure has been designed as a suspension test, rather than as a carrier test This preference is partly a matter of philosophy, partly guided by practical disadvantages of the carrier procedures Suspension tests (i) avoid the variation in porosity and electric charge exhibited by different surfaces *Mallmann & Leavitt* (1948) and *Rogers et al* (1961) found that porous surfaces require higher disinfectant concentrations than smooth ones Suspension tests (ii) measure microbial kill alone, where as carrier tests are influenced by the detergent activity also Besides, (iii) the drying procedure inherent in preparing the carriers alters bacterial viability Drying increases kill of Gram negative microbes (*Stedman et al* 1954) whereas *S aureus* may become more resistant to disinfectants, *e g* ethylene oxide (*Nystrom* 1969, personal communication) Suspension type tests have been recommended amongst others by the Public Health Laboratory Service Committee on the Testing and Evaluation of Disinfectants (1965) and by *Kelsey et al* (1965) Suspension techniques have the advantage that they are relatively rapid, convenient to handle, and stand a better chance of being reasonably reproducible

The present technique was introduced in an attempt to construct a better procedure, it fulfils four of the five points outlined by *Sykes* (1965) for an "ideal" test, *viz*

(1) A more definitive end point representing a kill of somewhat less than the usually measured 100 per cent kill,
(2) Using a wider range of test organisms,
(3) Adequate disinfectant quenching systems to counteract bacteriostatic carry-over, and
(4) Inclusion of organic matter

In addition, to reduce sampling error which was considered greater after short times of exposure, *Sykes* (1965) also preferred a long disinfection period of 30 minutes We employed a 5 minute reaction time The shorter disinfection period seems entirely realistic (BS 541 1934, BS 808 1938, *Jensen & Jensen* 1933, *Bass & Stuart* 1968) Although the time of disinfection in practice can usually be extended, the advantage of a 5 minute period is that it is more relevant to situations where a rapid disinfection is needed Indeed, 5 minutes exceeds the contact time encountered in many instances, but corresponds better to the period it takes for disinfectants to dry after application to floors, walls, table tops, beds, or similar objects In fact, some workers even think 5 minute exposures too long *Weber & Black* (1948), for instance, for a survivor curve procedure employed intervals of 30 seconds and 2 minutes There is no reason why samples may not also be taken after 30 minutes, but that requires a rather time consuming extension of the experiment which we found unrealistically elaborate, and after comparisons of these results with those obtained by the *Kelsey et al* (1965) procedure also less interesting We did not find 30 minute ED s more reproducible than the 5 minute ED s

Let us analyse *Sykes* (1965) first point A complete kill end point is to be avoided because it is unrealistic and cannot be measured accurately as becomes obvious from the statistical laws of variation and probability A complete kill end point actually determines growth in only a small aliquot—*e g* a loopfull or 0 1 ml Sampling variation is a concern when there is less than one survivor per aliquot The Poisson distribution

makes 29 negative subcultures possible even when the real survival is 1/20 A complete kill end point test commonly used is the AOAC use dilution test (*Bass & Stuart* 1968) where ten negative replicates are considered proof of complete kill It has been determined that to provide a result within a 95 per cent confidence limit, the suspension tests must be repeated considerably more than is commonly recognized (*Stuart* 1968) Other such tests are the *Kelsey et al* (1965) capacity test and the phenol coefficient procedures (RW, CM, AOAC)

Sykes (1965) preferred an end point of less than 100 per cent kill This amounts to defining an inactivation factor (= reduction factor) the relationship between initial number of germs and number of survivors expected after the killing procedure (*Christensen* 1964) This is done in assessing the kill achieved by heat or radiation The inherent condition is that dose kill curves are exponential rendering straight line relationships in semilogarithmic plots For practical purposes the deviation from the exponential law found in some instances may be considered insignificant The resulting errors are minor compared to the considerable advantage of straight line statistics

The exact size of the inactivation factor obviously depends on test design The suitability of the present 99 99 per cent kill as end point is derived from the starting number of cells After the various dilutions and transfers an inactivation of 10^4 results in 100 colonies Sampling will inevitably lead to unduly large plating errors with higher end point kills The bacterial inactivation determined in previous quantitative procedures has varied considerably *Jordan & Jacobs* (1944ab) determined 99 999999 per cent kill by extrapolation starting with approximately 3 10^9 bacteria per ml This implies only a single digit survivor cell number per ml of the starting suspension *Mossel* (1963) chose a 99 999 per cent point A 99 99 per cent point has been employed previosuly by *Baker & McClung* (1939) and was considered the most desirable level by *Davis* (1960) *Johns* (1947) and *Sykes* (1965) have held 99 9 per cent kill or greater to be reasonable A 99 9 per cent point was used by *Goetchius & Botwright* (1950), *Stedman et al* (1954), and by *Levine et al* (1926/27) *Levine et al* studied the dynamics of inactivation with time and found the inactivation velocity (k) to vary so considerably that they found 99 9 per cent kill suitable In studying spore inactivation *Myers* (1929) preferred 99 per cent kill considering smaller counts inevitably to cause larger experimental error *Withell* (1942) had more confidence in a 50 per cent kill end point This resembles the determination of IC_{50} (50 per cent inhibitory concentration) of antibiotic testing Although this point appears useful for comparisons of the efficacy of disinfectants or the sensitivity of test strains, it is useless for use dilution evaluations because the surviving cells inevitably include the most resistant cells

For comparisons of different disinfectants beside complete kill end points or employment of inactivation factors other measures have been employed *Hobbs & Wilson* (1942) found that the inactivation velocity value (k) determined at the middle of the disinfection process was useful for comparisons when the number of cells at the outset was the same in experiments to be compared *Berry & Cook* (1950) used a nephelometric end point and found approximately a rectilinear turbidity reduction with time Loss of enzyme activity or reduction of oxygen uptake as measures for disinfection has been reviewed by *Sykes* (1965)

Sykes (1965) called for the employment of a wide range of test organisms Our findings underline the variation in resistance found within the same species and the importance of selecting indicator strains only after a preceding scrutiny of a larger collection Pseudomonads proteus klebsiella and mycobacteria are the most resistant Whether to include spores or fungi is largely a matter of philosophy

The third point on *Sykes* (1965) list of prerequisites for the ideal test design is self

explanatory but is all too often disregarded in reports on disinfectant testing The inclusion of organic matter has been urged to implement the practical relevance of disinfectant testing Yeast has been used here because it is easy to prepare has precedence (BS 808 1938) and is easier to standardize than serum milk or feces

A comment is in order regarding the type of water for disinfectant diluent Electrolytes inactivate some disinfectants Therefore in many geographical locations it is worth while to employ water of standard hardness e g the World Health Organization (WHO) standard of 342 ppm The Disinfection Reference Laboratory at the Central Public Health Laboratory Service uses a salt content of 300 ppm (*Kelsey* & *Sykes* 1969) In Norway public water rarely exceeds 10 ppm hardness accordingly we have employed sterilized tap water for making our disinfectant dilutions In addition to these standardizations it is of paramount importance that a stable pH is maintained (*Stuart et al* 1950) and crucial that the temperature is sharply fixed The latter can only be satisfactorily achieved by special water baths with competing heating and cooling coils

One point of basic interest in our studies has been the comparison of the present quantitative technique with the capacity use dilution test (*Kelsey et al* 1965) used by us previously (*Bergan* & *Lystad* 1971) The concentrations of QAC s and halogens which correspond to the ED s of the present technique are often more than 10 times less than the use dilutions suggested by the capacity test The liquid milieu for the tests in our laboratory has been the same for both tests This supports the conclusion that the *Kelsey et al* procedure incorporates a considerable margin of safety We consider the ED s of the British Standard quantitative technique to represent minimum performance concentrations The BS test has the advantage that it considers the sampling error and allows valid dynamic comparisons between strains and disinfectants

After comparisons of *different transformations* of dose response the log probit procedure and the unweighted regression were found most satisfactory Consequently our conclusions for the efficacy of disinfectants have been based on the ED s calculated by log probit procedures although the log log transformations would also be acceptable as has been suggested previously (BS 3286 1960)

As for the disinfecting power of the chemicals included in this investigation the weak antimicrobial effect of the QAC s is illustrated by the present as well as our previous results (*Bergan* & *Lystad* 1971a) Preparation A included EDTA which renders the cell wall more permeable, probably through binding of bivalent ions causing a lowering of the ED s The chloramine rendered surprising ED s considering that the assay system was grossly contaminated by organic matter The results with picloxidine benzalkonium octyl phenoxypolyethanol makes this brand more acceptable than appeared after our previous results (*Bergan* & *Lystad* 1971) from which it evolved that 6 25 per cent of the commercial brand was necessary For this preparation *Kelsey* & *Maurer* (1967) suggested 2 25 per cent for soiled conditions The results obtained with glutaraldehyde indicate that 2 per cent renders a totally insufficient margin of safety This was also indicated by experiment evidence with other strains due to the lack of a suitable inactivator the membrane filter method was used This could not be as sharply defined regarding time that satisfactory rectilinearity resulted in more than the experiments listed

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A TRANSFORMED YULE CORRELATION COEFFICIENT EMPLOYED IN NUMERICAL GROUPING PROCEDURES ON BACTERIOPHAGE LYTIC SPECTRA

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A transformation of the Yule correlation coefficient (S_{φ}) yielding a range for the values from 0 to 100 was used for the assessment of the relationship between pseudomonas typing bacteriophage lytic spectra The S_{φ} compared well with the Jaccard Sneath similarity coefficient (S_{JS}) the use of which was reported in a previous communication Correlation analysis of these two similarity statistics rendered $r = 0.8834$ The unweighted pair group average cluster analysis based on S_{φ} ((S_{φ}) UWPGA) has a higher cophenetic correlation than the corresponding weighted pair group procedure rendering correlation coefficients of 0.8122 and 0.7450 respectively However the (S_{φ}) UWPGA did not have as high a correlation to the similarity matrix as achieved for the same cluster procedure based on S_{JS}

In a previous communication attempts have been made to apply numerical allocating procedures to pseudomonas bacteriophages in order to group them according to similarities in their phage lytic spectra The grouping procedures used there (1) have been commonly used in numerical taxonomy and were based on the Jaccard Sneath index (S_{JS}) and the simple matching coefficient (S_{SM}) However for phages lysing less than 15 per cent of the bacteria, a linear relationship between their maximum value of S_{SM} and per cent lysis was identified The S_{SM} also was associated with a considerable phenogram chaining Although by far not as pronounced for S_{JS}, also with that similarity statistic was there a tendency towards higher maximum similarity index values for phages with more lytic reactions (1) Consequently it was desirable to investigate the properties of yet another affinity statistic

Contingency table data being handled well by correlation coefficients the fourfold point correlation coefficient of Yule was used and transformed to render values from 0 to 100 corresponding to the range for S_{JS} and S_{SM} The purpose of the present paper is to evaluate the transformed Yule correlation coefficient (S_{φ}) as a basis for grouping pseudomonas bacteriophages according to their lytic spectra

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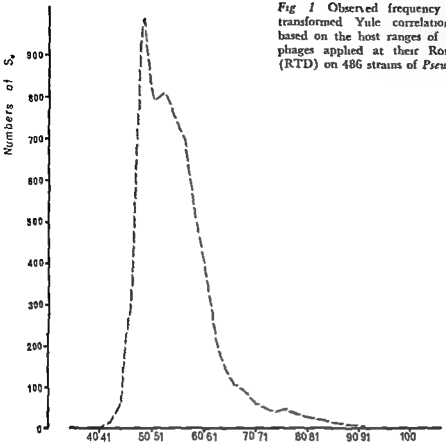


Fig 1 Observed frequency distribution of the transformed Yule correlation coefficient ($S\varphi$) based on the host ranges of 113 typing bacterio phages applied at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa*

MATERIALS AND METHODS

Microorganisms and Bacteriological Procedures

Details on the bacteriophages, bacterial strains, propagation procedures, typing methods, media, numerical and statistical methods and explanation of terminology used are given elsewhere (1)

Numerical Procedures

The Yule correlation coefficient (21) may be derived from the well known product moment cor relation coefficient (r), given in any standard text book of statistics (24) (see Appendix)

As a correlation coefficient, φ varies within the interval —1 to +1 φ was transformed by the formula

$$S\varphi = 50 + 50\varphi$$

for the affinity between items 'I' and 'J' to obtain the same range of variation as S_{JS} and S_{SM}

Certain characteristics of the transformed Yule correlation coefficient may be described After transformation according to the above equation for $S\varphi$,

$S\varphi_{hm} =$ 100 for best similarities and
$S\varphi_{\ im} =$ 0 for lowest affinities

as is the case also with S_{JS} and S_{SM} $S\varphi$ has no meaning unless $n_{jk} \neq 0$ and $n_{jk} \neq 0$, or $n_{jk} \neq 0$ and $n_{jk} \neq 0$, i e there must be non zero values along at least one diagonal of the ONU pair contingency table Consequently, in the unlike ly event of exclusively either all positive or all negative characters and complete identity for all characters ($n_{jk} = n$ or $n_{jk} = n$) $S\varphi = 100$ If, on the other hand, for $n_{jk} = n$, or $n_{jk} = n$, one obtains $S\varphi = 0$ It shall be noted that, like in the S_{SM} index, both positive and negative matches are entered in the $S\varphi$ numerator This results in the more desirable situation where both positive and negative reactions are considered for ONU comparisons

RESULTS

Distribution of the Sφ Similarity Indices

The frequency distribution of the $S\varphi$ values is shown in Fig 1, the mode lies at the $S\varphi$ interval of 46-49 The mean $S\varphi$ is 55 19

Cluster Analysis

Figs 2 and 3 demonstrate the WPGA and the UWPGA based on the $S\varphi$* The bifurcations of the phenograms are distributed throughout the whole range of 50–100 per cent but usually there is a fairly good cluster distinction Comparison of the two dendrograms shows that the higher the affinity level the more similar do the cluster patterns tend to be e g clusters 35–66 18–81, 10–73, 1–68 8–109 17 85 22–67 26–34 Clusters 74–87 and 74–113 in the UWPGA and WPGA respectively contain the same elements The groups 22–67, 26–34 and 63–72 are clustered together in both dendrograms but are joined in slightly different ways The 26–34 cluster incidentally consists only of phages from the Meitert typing set (see Table 1, reference (1))

The 45–49 group is joined by ONU 53 at the 58 per cent level in both diagrams, but the 42–43 pair which joins these at the same level in the UWPGA belongs to an entirely different set in the WPGA Generally, in the two dendrograms at the lower levels unions are achieved by different clusters

The relationship between the two phenograms is shown in Table 1 to be r = 0 8473

TABLE 1 *Correlation between the Transformed Yule Correlation Coefficient ($S\varphi$) its Weighted and Unweighted Pair Group Average Cluster Analysis (WPGA and UWPGA) Cophenetic Values the Jaccard-Sneath Similarity Index (S_{JS}) the Simple Matching Coefficient (S_{SM}) and the WPGA and UWPGA Cophenetic Values Based on the S_{JS} and S_{SM}*

	$S\varphi$	[$S\varphi$] WPGA	[$S\varphi$] UWPGA
$S\varphi$	—		
[$S\varphi$] WPGA	0 7450	—	
[$S\varphi$] UWPGA	[illegible]	[illegible]	—
S_{JS}	0 8834	0 6955	0 8119
[S_{JS}] WPGA	0 7501	0 7854	0 8382
[S_{JS}] UWPGA	0 7598	0 6969	0 8547
S_{SM}	0 1627	0 1909	0 0974
[S_{SM}] WPGA	0 1653	0 2588	0 1885
[S_{SM}] UWPGA	0 0158	0 1327	0 0466

Subject of the analyses was a Q matrix analysis of the host range of 113 typing bacteriophages applied at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa*

* Brackets around a similarity index denotes its matrix

Relationships between $S\varphi$, [$S\varphi$] WPGA and [$S\varphi$] UWPGA

Figure 4 shows that there is no distinct interdependence between the maximum $S\varphi$ value for a phage and the percentage of the bacterial strains lysed by that phage, although phages which lyse more bacterial strains tend to have higher maximum $S\varphi$ The relationships between the $S\varphi$ matrix and the two clustering procedures were examined by covariance analysis (Table 1) The unweighted procedure exhibited the highest correlation to $S\varphi$ (r = 0 8122) The mean cophenetic value for the [$S\varphi$] UWPGA phenogram was 55 63 and for [$S\varphi$] WPGA 54 92 The higher correlation of the former phenogram to the similarity matrix is also reflected in a smaller difference between the mean similarity index value and the mean cophenetic value for the [$S\varphi$] UWPGA than for the [$S\varphi$] WPGA The relative similarity of the two dendrograms was mediated by a high correlation coefficient between cophenetic values derived from the two clustering procedures (r = 0 8473) The cophenetic variance between the two is demonstrated in Fig 5

Correlation between $S\varphi$ S_{JS} and S_{SM} with the Phenograms Derived Therefrom

The results of the correlation between each of $S\varphi$ S_{JS}, and S_{SM} and the cluster procedures based on them are shown in Table 1 Two-way distributions between $S\varphi$ on the one hand and S_{JS} and S_{SM} on the other are shown in Figs 6 and 7 It is evident that [$S\varphi$] and [S_{JS}] are the two most similar affinity coefficient matrices The correlation coefficient between [$S\varphi$] and [S_{JS}] is 0 8834 whereas the correlation of [$S\varphi$] to [S_{SM}] is only 0 1622

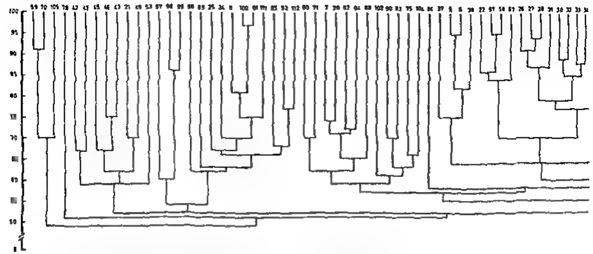


Fig 2 Phenogram achieved by the weighted pair group average cluster analysis (WPGA) of the host *monas aeruginosa* based on the transformed Yule correlation coefficient (Sφ) The ordinate ■ divided is indicated by 50, +1 by 100, and —1 by zero on the figure scale

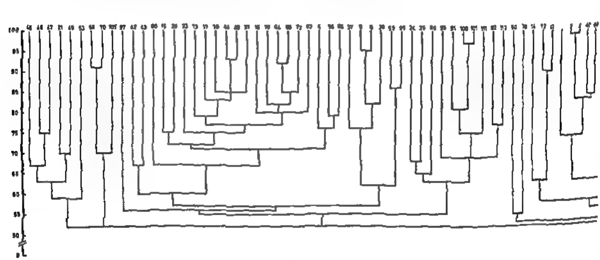
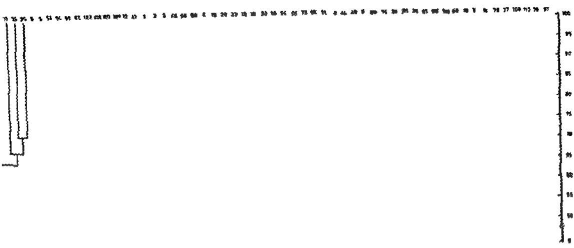


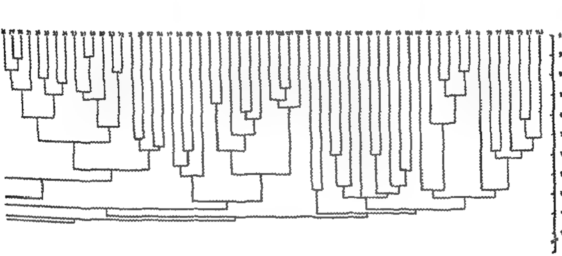
Fig 3 Phenogram for the unweighted pair group average cluster analysis (UWPGA) with Sφ based on

Since S_{SM} ■ misleading in this context (1), and the weighted cluster analysis has been shown to be inferior in all instances, a comparison of the [Sφ] UWPGA and the [SJS] UWPGA dendrograms becomes the most interesting Similar bifurcation patterns are exhibited by the groups 59–105, 50–51, 60–71, 1–68, 14–66, 7–84, 8–109, 74–113, and in 37–85, except that ONU 17 which ■ added to the cluster 15–85 at the 63 per cent level in [Sφ] UWPGA, in [SJS] UWPGA clusters with ONU 94 at the 20 per cent level Although different intragroup patterns are exhibited, the groups 96–112, 42–53 in [Sφ] UWPGA and 53–49 in [SJS] UWPGA, 80–86 in [Sφ] UWPGA and 4–86 in [SJS] UWPGA, and 75–104 in both, contain the same ONU's

In the second group, though, ONU 43 is extra The ONU s 12–41 and 98–99 exhibit fairly high affinities in both dendrograms and are, together with 97 and 102 interconnected differently at the lower levels The ONU s 17, 43, 37–74, 78, 94, and 110 exhibit dif ferent affinities in the two diagrams Thus, although there are a number of differences in



ranges for 113 typing bacteriophages at their Routine Test Dilution (RTD) on 486 strains of *Pseudo-*
;; 100 units corresponding to the correlation coefficient range −1 to +1, zero correlation consequently



the same data as in Fig 2 Explanation of the ordinate scale in Fig 2

detail, the two clustering diagrams are fairly similar This is reflected in a correlation coefficient of r = 0 8547 between the UWPGA cophenetic values based on S_{φ} and S_{JS} (Fig 8)

DISCUSSION

Since two coefficients of association have already been employed, S_{JS} and S_{SM}, and different results were obtained, it was found necessary to investigate yet another matrix of similarity Distance coefficients have received favourable evaluation for many problems (15) For bivariate scoring systems the mean distance (21) becomes $\bar{d}_{j} = (1-S_{SM})^{\frac{1}{2}}$ Being thus related to one of the previously used indices this does not entail anything particularly new To employ any of the distance transformations based on similarity indices for factor analysis or multidimensional scaling would become a tremendous computational task with as many as 113 ONU's

Correlation coefficients have been employed frequently in Q matrix factor analysis in taxonomy (21) ecology (5), and psycho-

logy (22). A correlation coefficient was used, e.g. by *Rohlf* (15) and by *Boyce* (2) for multivariate state characters. Boyce (2) found that when compared with (a) cosine of the angle between two forms, (b) mean character difference (M.C.D.), (c) mean square distance, and (d) Penrose's shape coefficient, the relationship patterns which

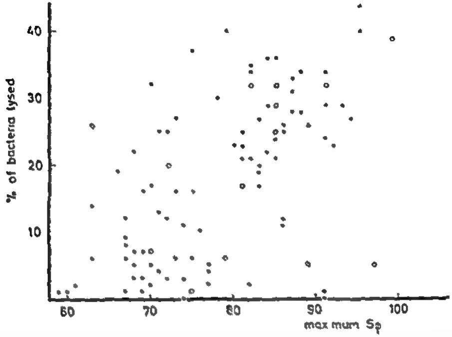
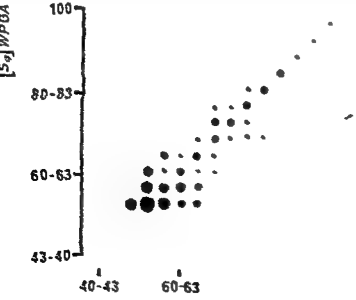


Fig. 4. Relationship between the highest value of the transformed Yule correlation coefficient (maximum Sφ) for each of 118 typing bacteriophage lytic spectra and the percentage lysed among 486 strains of *Pseudomonas aeruginosa*. Explanation of the abscissa as for the ordinate Fig. 2. Key of symbols: • = 1 observation, ○ = 2 observations, Δ = 3 observations. The correlation coefficient between the two variables is r = 0.5394, the level of significance being α < 0.001.



corresponded most closely to that conceived by intuitive procedures were indicated by the correlation coefficient

Minkoff (12) for various reasons considered correlation coefficients to be less meaningful *Minkoff* worked with antropometric data and for these preferred the Mahalanobis generalized distance (D^2) which was originally developed for exactly such data

A 2×2 table describing the relationship between two particular ONU's may be evaluated by correlation coefficients The fourfold point correlation coefficient (r_{ij}) and the product moment correlation coefficient (r) which are related to the S_φ used presently, have previously been used in taxonomy *Yule & Kendall* (24) described a two way correlation coefficient which in our notation reads

$$r = \frac{n(n_{JK} - n_J n_K)}{(n_J n_K n_j n_k)^{\frac{1}{2}}}$$

The Yule correlation coefficient described in the Appendix appeared theoretically preferable because both positive and nega-

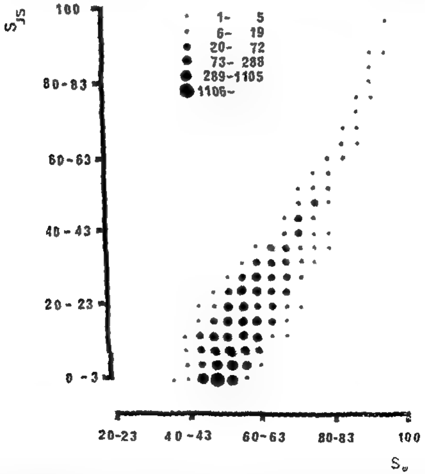


Fig 6 Two way frequency distribution for the transformed Yule correlation coefficient and the Jaccard Sneath similarity index (S_{JS}) for the host ranges of 113 typing bacteriophages at their Routine Test Dilution (RTD) on 486 strains of *Pseudomonas aeruginosa* The scale of the abscissa as in Fig 2, the scale of the ordinate in per cent similarity Each cell of the graph has been obtained by uniting the frequency at each point as indicated by dots of varying diameter according to the key in the upper left hand corner The correlation coefficient between the two variables is r = 0 8834

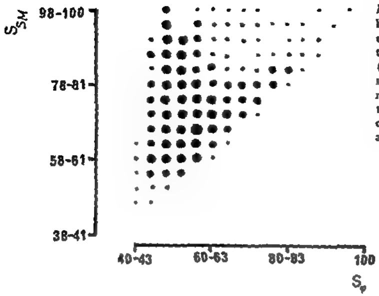


Fig 7 Two-way frequency distribution for the transformed Yule correlation coefficient (S_φ) and the simple matching coefficient (S_{SM}) The scale of the abscissa is explained in Fig 2, the ordinate indicates per cent similarity Key to graph symbols in Fig 6 The correlation between the two variables is $r = 0\ 1622$

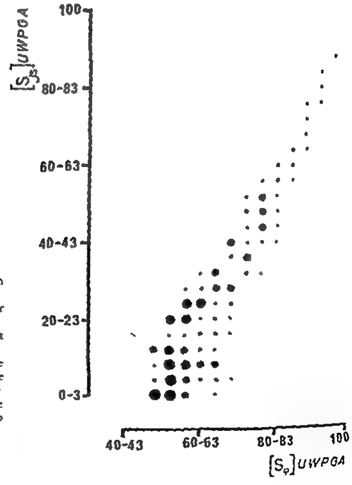


Fig 8 Two way frequency distribution between cophenetic values of the unweighted pair group average cluster analysis (UWPGA) based on the transformed Yule correlation coefficient ($S\varphi$) (see Fig 3) and the Jaccard Sneath similarity index (S_{JS}) The scale of the abscissa explained in Fig 2 The ordinate indicates per cent similarity Graph symbols explained in Fig 6 The correlation coefficient between the two variables is $r = 0\ 8547$

tive matches were included in the numerator. The $S\varphi$ values appeared more independent of the percentage of bacterial strains lysed (Fig. 4) than Sjs (1), having only a weakly suggested tendency to that effect.

The question now is how useful the $S\varphi$ is in the evaluation of bacteriophages according to their lytic spectra. Unfortunately, no exact procedure exists for evaluating how well or how badly the $S\varphi$ has managed the grouping. A t-test, for instance, is inapplicable for theoretical reasons. It is conceivable that the ONU's here are not entirely stochastic and the experimental data diverges from a normal distribution. Among the 113 phages some share a common origin, but have been received here from different sources. Some of these are suitable as controls of sorts as to whether the clusterings achieved are reasonable. The classification of such ONU's in both $S\varphi$ dendrograms compare well with preconceived subjective ideas of their present interrelationships.

The level which indicates stronger ONU similarities as judged by the over-all patterns of relationships, known phage history, and derivation of the phage strains corresponds roughly to 75–80 per cent for the phenograms based on Sjs and 90–95 per cent for $S\varphi$ dendrograms. Analogous to the discussion of *Sokal & Michener* (19), it is likely that the phages are not either unrelated or related *per se* but simply share varying degrees of affinity reflecting the evolutionary trends of gradual divergence from one phage lytic spectrum into another.

In lieu of correlating the similarity matrix to the data matrix, it is useful to compare $S\varphi$ with other coefficients of affinity. Correlation analysis for $S\varphi$ and Ssm (Fig. 7) rendered r = 0.1622. Since Ssm was to some extent misleading when applied to the present bacteriophage lytic spectra (1), this r did not particularly relate to the validity of $S\varphi$, but is still important because *Sokal & Michener* (19) reached essentially identical affinities by the product moment correlation coefficient and Ssm. Between $S\varphi$ and Sjs, however, there was a more satisfactory correlation (r = 0.8834) as is illustrated in Fig. 6. Cophenetic analysis of relevant phenograms (Fig. 8) rendered r = 0.8547 which is high considering that it refers to as many as 6328 elements. These findings tend to support the validity of $S\varphi$. When examining the phenograms based on $S\varphi$ and Sjs, it was apparent that $S\varphi$ has produced a better distinction between clusters, although the difference in cluster composition between the dendrograms of the two indices is relatively minor.

The distortion caused by the clustering relative to the $S\varphi$ similarity matrix has been evaluated by correlating it to the cophenetic values of [$S\varphi$] UWPGA and [$S\varphi$] WPGA (Fig. 5). The UWPGA had a better correlation to the $S\varphi$ than did the WPGA: r = 0.8122 and r = 0.7450 respectively (Table 1). It is important to note that a similar situation ocurred for both Sjs and Ssm, the best correlation being obtained for [Sjs] UWPGA (r = 0.9303) (1).

The general conclusion that UWPGA was the clustering procedure which processed the similarity matrices most faithfully, is most significant. This is in accordance with theoretical considerations presented previously (1) which emphasized that large clusters would tend to become more heterogenous when the latest ONU's counted weighted more than the clustered ONU's at earlier clustering cycles (the weighted procedure). This finding agrees also with earlier studies made with smaller ONU matrices. *Boyce* (2, 3) found r = 0.890 for UWPGA and r = 0.861 for WPGA using the product moment correlation coefficient as the similarity index. It is interesting that *Boyce* (2) for the WPGA found the same cophenetic correlation as *Sokal & Rohlf* (20) who found 0.86. A low cophenetic value was achieved by the UWPGA based on r and average distance in another study (8) where factor analysis was however not found satisfactory either. When these clustering procedures were first employed, it was thought that the weighted variable group procedure would be preferable (19). Our conclusion is in agreement with

the present experience of *Sneath* (17) which is that the UWPGA usually yields the highest cophenetic correlation

In all, in the present and a previous communication (1), the typing bacteriophages have been grouped by six different procedures, using three coefficients of affinity (S_φ, S_{JS}, and S_{SM}) and two clustering procedures (WPGA and UWPGA) Although the [S_{JS}] UWPGA or [S_φ] UWPGA in this context appeared to be the phenograms on which conclusions could be drawn the circumstance that no two dendrograms are alike requires comment Although it could be taken to indicate a kaleidoscopically indeterminate distinction between all the presently investigated pseudomonas typing phages certainly, the phenogram differences are at least in part due to methodological differences To eliminate possible misunderstanding it should at this point, be mentioned that on the basis of these cluster diagrams no predictions should be made regarding the phenetic or phylogenetic interrelationships between the phages, because these affinities have been based on lytic spectra only

It is the rule that different classifications are achieved by different numerical taxonomic methods (2 3, 10 13, 19) Indeed, this state of affairs has been adopted by the opponents of numerical taxonomy as proof of inferiority for these procedures compared to empirical methods and philosophy for grouping In numerical grouping however, the problem generally is to select the most suitable procedures for solving the problems at hand

The clustering procedures contain an unavoidable bias inherent in the very nature of the starting points These are determined by the (chance) existence of a pair of similar ONU s Addition of ONU s to induce other affinity constellations will lead to different starting points. This *a priori* and unavoidable bias is reminiscent of the Jancey technique (9) in which the starting points are arbitrarily selected The consequences of omitting ONU s however, has been minimal (19)

It is important in a numerical grouping study to know the extent of interdependence for the characters employed *In casu*, there is a considerable possibility that several bacterial strains from the same physical source (ward hospital) are descendants of the same original strain Still, the study of more than one strain of a group with identical characteristics probably has little less predictive value for the estimation of ONU affinity than the scrutiny of only one such strain (characteristic) An overrepresentation or underrepresentation of a given bacterial type, though will affect the calculated similarity matrix The degree of interrelationship between the pseudomonas strains is not readily assessible except by typing procedures but employing such would render a circular documentation

Consequently it is interesting to note how resistant a grouping pattern is to manipulations among the characters *Rohlf* (14) and *Fisher* & *Rohlf* (7) found that randomized feature selection only caused minor differences in the similarity index values Differences were least for ONU s with the lowest affinity of other items Similar findings were made by *Ehrlich* (6) However, classifications based on different sets have differed when the traits were somehow not related Distinct insect classifications were reached for entirely larval or entirely adult characters (13) or for head and non head traits (11)

The results reached by present numerical procedures are not final The employment of *a priori* selected principles for grouping is akin to the Aristotelian approach to logical division whereby sets of items are defined by their essence This means that certain prerequisites are mandatory and sufficient for an object to belong to a given class (2 4) This substitutes the empirical relatively undefinable subjectivity involved in matching *likes e g* in classical taxonomy Although not a final judge of affinities in spite of mathematical formality the computer procedures are helpful aids

APPENDIX

The transformation of the product moment correlation coefficient to the Yule correlation coefficient (21) is shown below

$$r = \frac{\Sigma x_i y_i - n \dfrac{\Sigma x_i}{n} \dfrac{\Sigma y_i}{n}}{\sqrt{\left\{ \Sigma x_i^2 - n\left(\dfrac{\Sigma x_i}{n}\right)^2\right\} \left\{ \Sigma y_i^2 - n\left(\dfrac{\Sigma y_i}{n}\right)^2\right\}}} \qquad (i)$$

where x_i and y_i are to denote the score of the reactions for phages "J" and "K", such that a positive reaction scores 1 and no reaction scores 0

Consequently $\Sigma x_i y_i = n_{jk}$

$$\Sigma x_i^2 = \Sigma x_i = (n_{jk} + n_{jK})$$

$$\Sigma y_i^2 = \Sigma y_i = (n_{jk} + n_{Jk})$$

The n-subscripts are as defined in Sokal & Sneath (21) n_{jK} is the number of positive matches n_{jk} is the number of reactions where phage J has positive reactions and phage K has negative reactions, n_{Jk} denotes the number of reactions where the first phage has negative and the second phage positive reactions, and n_{JK} is the number of negative reactions in common After substitution of these expressions in equation (i) and multiplication of numerator and denominator with $n = (n_{jK} + n_{jk} + n_{JK} + n_{Jk})$ one obtains

$$r = \frac{n_{jK} n_{Jk} - n_{jk} n_{JK}}{\sqrt{(n_{jk} + n_{Jk})(n_{JK} + n_{jK})(n_{jk} + n_{jK})(n_{Jk} + n_{JK})}} \qquad (ii)$$

which by insertion of the commonly employed expressions for the sums of rows and columns in a 2×2-table (21) becomes

$$r = \varphi = \frac{n_{jk} n_{JK} - n_{Jk} n_{jK}}{\sqrt{n_j n_J n_K n_k}} \qquad (iii)$$

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MICROBIOLOGICAL ASSAY OF JOSAMYCIN

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A sensitive bioassay for josamycin was obtained by an agar cup procedure using *Micrococcus luteus* CQ 70 ml Mueller Hinton Medium (Difco) per 14 cm diameter plates, 5 hours pre diffusion and over night incubation at 37° C This procedure allows measurements in the range of 0 01–4 0 µg/ml josamycin base Josamycin activity was enhanced by high pH values NaCl and $NaHCO_3$ had little influence on josamycin activity, whereas $CaCl_2$ was markedly inhibitory Josamycin base was relatively stable in acid milieu No inactivation was found after 6 weeks in serum at −70° C, or for 3 hours either in a pH 7 0 phosphate buffer solution or in a simulated intestinal fluid T S of the United States Pharmacopeia (USP XVIII) Only 35 per cent inactivation was found after 3 hours storage at room temperature in a simulated gastric fluid (USP XVIII)

Macrolide antibiotics have shown their versatility in the treatment of Gram positive microbes primarily in penicillinase producing strains of *Staphylococcus aureus* Macrolides constitute an alternative for patients with penicillin allergy Recently, a new macrolide, josamycin, has been isolated Its effect against mycoplasma (4, 5 9) occasionally surpasses that of erythromycin (6) Josamycin is more effective against staphylococci than spiramycin or oleandomycin (4) Although cross resistance is usually found about 40 per cent of erythromycin resistant staphylococci are sensitive to josamycin (9) The prospect of such a new compound of reportedly low toxicity has prompted our interest in josamycin

Motivated by our involvement in the pharmacodynamic characterization of the drug, the basic concern of the present report is the establishment of an accurate and sensitive assay procedure for josamycin

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MATERIALS AND METHODS

Bacterial Strains

As indicator strains were used *Micrococcus luteus** CQ (kindly supplied by Mr L Tybring, Løvens kemiske fabriker Copenhagen Denmark) *Bacillus subtilis* ATCC 6633 *Staphylococcus aureus* ATCC 6538p *Streptococcus pneumoniae* 22 and *Corynebacterium hoffmanni* 26 (received from Mr L Magni Astra AB Sodertalje, Sweden) For the use as indicators these bacteria were cultured on blood agar plates at 37° C over night

Chemicals

Trishydroxymethylaminomethan was produced by Koch Light Laboratories Ltd Colnbrook Eng land All other chemicals were of *pro analysi* grade and from Merck AG Darmstadt Germany The josamycin base reference standard was 97 3 per cent pure of lot No JC 21 generously supplied by Astra

* The name *M luteus* is used instead of *Sarcina lutea* since the genus *Sarcina* presently should encompass only anaerobic strains

AB, Sodertalje, Sweden For dilutions of 10 mg per ml ethyl alcohol was used as solvent Concentrations of 100 μg/ml or less were made in phosphate buffer of pH 7 or in pooled serum

Culture Media

The media used were Mueller Hinton Medium (Difco) (MH), Diagnostic Sensitivity Test Agar (Oxoid) (DST), Antibiotic Assay Medium No 5 (Difco) (AA5), and Antibiotic Assay Medium No 11 (AA11) The pH values were controlled *after* sterilization

Microbial Sensitivity

The determination of the josamycin concentration inhibiting the growth of 50 per cent of a bacterial population (IC_{50}) was carried out by a modified Spearman Karber method (3 8) Twofold dilutions of josamycin base in MH medium were employed The inoculum corresponded to a 1 200 dilution of an over night 37° C broth culture

Antibiotic Assay Procedure

An agar cup technique was employed whereby 10 mm cylinder holes were punched out of the agar and filled with standards and specimens with unknown josamycin contents The indicator strain suspensions were made from over night 37° C blood agar cultures treated on a Vortex mixer and standardized by means of a Coleman Junior Spectrophotometer in Penassay Broth (Difco) to an optical density (O D) of 0 3 One ml of the bacterial suspension was mixed with 100 ml melted agar medium of ca 50° C Josamycin standards were made in pooled human serum To minimize differences in temperature gradients during the 37° C over night incubation the plates were not stacked on top of each other The suitability of media and bacteria was investigated in double agar layer plates of 14 cm diameter with a 50 ml nutrient layer and a 20 ml top layer inoculated with the bacterial indicator strain Each assay was done in triplicate Inhibition zones were measured to an accuracy of 0 2 mm by an antibiotic zone reader Standard curves were obtained by the least squares method The Y axis exhibits the standard values and the X axis the mm inhibition zones

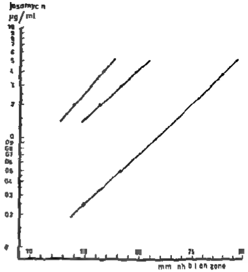

Fig 1 Standard lines for josamycin base with *Micrococcus luteus* CQ (x——x), *Bacillus subtilis* (o——o), and *Staphylococcus aureus* (——) as test organisms on Mueller Hinton Medium (Difco), pH 7 3

RESULTS

Choice of Assay Medium

Comparison between the media MH, DST, AA5, and AA11, showed the MH medium to yield the most sensitive system with both *M luteus* and *S aureus* The standard curves achieved with the different media are listed in Table 1

Choice of Assay Microbe

The results for IC_{50} in the MH medium for 5 bacterial strains are shown in Table 2 The standard curves in Fig 1 show that *M luteus* rendered the most sensitive assay procedure With a given standard concentration of josamycin, *M luteus* exhibited larger in

TABLE 1 *Regressions of Standard Curves with Josamycin with* Micrococcus luteus *as Test Organism on Four Different Media*

Medium	Equation of regression x =
Antibiotic Assay Medium No 5 (Difco)	5 4951 + 13 4550 y
Antibiotic Assay Medium No 11 (Difco)	—2 9164 + 17 4071 y
Diagnostic Sensitivity Test Agar (Oxoid)	7 7812 + 12 6747 y
Mueller Hinton Medium (Difco)	9 5431 + 11 7980 y

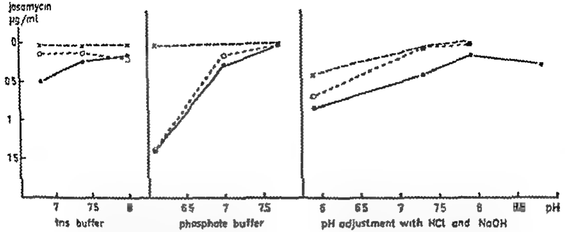

Fig 2 The influence of pH variations on bacterial sensitivity to josamycin base measured as IC_{50} (*i e* the drug concentration inhibiting the growth of 50 per cent of the bacterial cells) The strains examined were *Micrococcus luteus* (x——x), *Bacillus subtilis* (o——o), and *Staphylococcus aureus* (•——•) The pH value was recorded after sterilization of the medium Mueller Hinton Medium (Difco) was used as base medium.

hibition zones than the two other indicators The intercept of the *M luteus* line with the y axis accordingly corresponds to the lower

TABLE 2 *Sensitivity to Josamycin Base of 5 Bacteria Investigated for Their Suitability as Test Strains*

Microbe	IC_{50} (μg/ml)
Bacillus subtilis	0.90
Corynebacterium hoffmanii	0.02
Streptococcus pneumoniae	0.03
Micrococcus luteus	0.09
Staphylococcus aureus	0.42

Comparisons made by agar dilution technique in Mueller Hinton Agar (Difco), pH 7.3

IC_{50} = That concentration which inhibits 50 per cent of the bacterial *population*

drug concentration There was a poor growth intensity of *S pneumoniae*, it was therefore difficult to read zone diameters The smallest zones of inhibition with *C hoffmanii* were difficult to measure accurately

Optimal pH for Josamycin

The antimicrobial effect of josamycin as a function of pH was tested with three bacterial strains (Fig 2) With the HCl/NaOH system, josamycin was most active in the alkaline range This tendency was marked for *B subtilis* and *S aureus* in phosphate buffer, but not for *M luteus* The tris buffer could only be employed within the pH range of 7–8 where the difference in josamycin activity were minimal, with a small difference

TABLE 3 *Influence of NaCl on Josamycin Activity Tested on Mueller Hinton Medium (Difco), pH 7.3*

NaCl addition (%)	IC_{50} (μg/ml) *B subtilis*	*M luteus*	*S aureus*	Regression equation for *M luteus* standard curve x
0	0.09	0.09	0.42	10.3012 + 10.0988 y
1	0.11	0.02	0.25	10.3870 + 9.8330 y
2	0.11	0.02	0.21	10.3539 + 10.0431 y
3	0.35	0.18	0.35	11.2524 + 9.4676 y

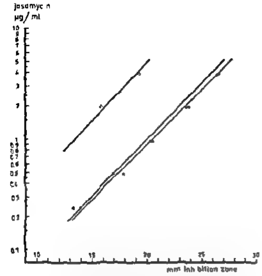

Fig 3 Standard curves of josamycin base achieved with Mueller Hinton Medium (Difco) with different pH values as obtained by adjustment of the NaOH/HCl contents 5 9 (o——o), 7 3 (——), and 7 9 (x——x) Test organism was *Micrococcus luteus*, pH values were controlled after the medium was autoclaved The regression equations for the respective curves are x = 3 8528 + 9 4649y x = 10 3012 + 10 0988y, x = 10 0668 + 9 7332y There was no growth on medium of pH 8 8

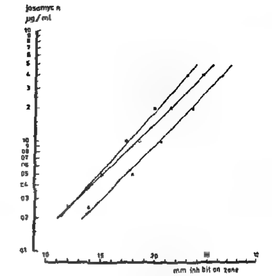

Fig 4 Influence of $CaCl_2$ on josamycin base standard curves achieved by *Micrococcus luteus* Legend x——x represents the Mueller Hinton Medium (Difco) (MH) at pH 7 3, o——o the MH medium with 0 01 M $CaCl_2$ added, and —— the MH medium with 0 05 M CaCl The regression equations for the respective curves are x = 10 3012 + 10 0988y, x = 7 9487 + 10 3313y, x = 8 8633 + 8 6369y

only for *S aureus* This ocurred for all systems used to adjust the pH of the growth medium HCl/NaOH, tris buffer, and phosphate buffer Accordingly, the higher pH values resulted in more sensitive assays (Fig 3) Optimal growth intensity of the test strain was seen within the pH range 6 5–8 0

The Effect of NaCl on IC_{50} and Assay Sensitivity

The influence of varying sodium chloride concentrations for antibacterial effect and assay sensitivity is shown in Table 3 With *B subtilis* and *M luteus*, the IC_{50} was highest with 3 per cent NaCl The effect on *S aureus* was at a maximum with 2 per cent NaCl added However, the assay was slightly more sensitive with 3 per cent NaCl The difference between other salt concentrations was insignificant Growth of *M luteus* was reduced on the medium with 3 per cent NaCl

The Influence of $NaHCO_3$ on Microbial Sensitivity and Assay Precision

Table 4 shows a negligible influence of 0 01 and 0 05 M $NaHCO_3$

Modification of Antimicrobial Effect by Calcium

It was found that $CaCl_2$ in the MH medium markedly reduced the effect of josamycin Accordingly, $CaCl_2$ increased the IC_{50} of

TABLE 4 *The Effect of Na HCO_3 on the Activity of Josamycin Tested with Mueller Hinton Medium (Difco), pH 7 3*

$NaHCO_3$ addition (M)	Regression equation for standard curve (*M luteus*) x =
0	10 3012 + 10 0988 y
0 01	9 7176 + 10 9624 y
0 05	9 8972 + 10 8628 y

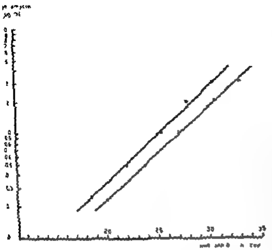

Fig 5 Influence of 5 hours prediffusion at 4° C anism was y supplied Sweden)

Legend o——o is the result with an —— without prediffusion

all 3 species tested and reduced the assay sensitivity (Fig 4, Table 5)

Medium Thickness and Prediffusion Influence on Assay Sensitivity

With *M luteus* as test microbe and plates with 70 ml medium, there was no appreciable difference in assay sensitivity whether the bacterial indicator infected the entire agar or only a 20 ml top layer The influence of 5 hours prediffusion at 4° C on the standard curve is shown in Fig 5 No difference in assay sensitivity was found between plates with 70 ml and with only 20 ml medium per 14 cm diameter plates

TABLE 5 *Effect of $CaCl_2$ on IC_{50} (Inhibitory Concentration for 50 Per Cent of the Bacteria) as Shown by Strains of Different Bacterial Species on Mueller Hinton Medium (Difco) pH 7 3*

$CaCl_2$ addition (M)	IC_{50} (µg/ml)		
	B subtilis	*M luteus*	*S aureus*
0	0 09	0 09	0 42
0 01	0 25	0 15	0 59
0 05	0 73	0 30	3 36

Josamycin Stability in Gastric and Intestinal Fluid

The stability of josamycin was tested in simulated gastric and intestinal fluids T S of the United States Pharmacopeia (USP XVIII) No reduction of activity occurred in the intestinal fluid, but a 35 per cent reduction was observed in the gastric fluid after 3 hours In the gastric fluid the activity was reduced by 15 per cent after 30 minutes and by 25 per cent after 1 hour No significant reduction of activity was noted after 4 hours in a pH 7 0 phosphate buffer Storage in pooled serum at —70° C for 6 weeks, or freezing and thawing three times a week did not affect josamycin activity

DISCUSSION

The serum concentrations reached after josamycin administration to healthy persons were relatively low Consequently, it was essential that the sensitivity of the assay procedure was optimal and that factors with an unfavourable effect on sensitivity were detected

M luteus CQ was the most suitable test strain for attaining a highly sensitive assay procedure prediffusion for five hours improved sensitivity

The composition of the medium often influences assay sensitivity Mueller Hinton Medium (Difco) was preferable to 3 other commonly used alternatives Because the contents of inorganic salts often vary from one medium to the other, the influence of 3 such biologically significant compounds were studied Neither sodium bicarbonate nor sodium chloride significantly altered the josamycin activity The inverse effect of sodium chloride on (a) IC_{50} and (b) assay sensitivity is notable In spite of the finding that 3 per cent NaCl was associated with a slightly higher IC_{50} the same concentration rendered the most sensitive assay Calcium chloride, on the other hand clearly inhibited the antibiotic action More than 0 05 M $CaCl_2$ also markedly inhibited gentamycin (7) This may be caused by a reduction in cell wall permeability

The effect of the pH values was clearly demonstrable Although josamycin was more active in the alkaline range, it did not seem to be quite as dependent on alkalinity as other macrolides, *e g* erythromycin This difference is discernible from the dissociation constants, since pK_a of josamycin [redacted] 7 1 whereas pK_a for erythromycin is as high as 8 8 (1) The results of these investigations indicated that the MH medium [redacted] well suited for routine assays

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INFLUENCE OF GLYCOPROTEIN FROM RED CELL MEMBRANES ON THE AGGLUTINATING AND LYMPHOCYTE STIMULATING ACTIVITY OF PHYTOHAEMAGGLUTININ

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Lymphocytes were incubated in the presence of each of two phytohaemagglutinin preparations One of these preparations (H PHAP) agglutinated red cells and a mixture of leucocytes and platelets The other preparation (L-PHAP) agglutinated leucocytes platelets but not red cells A soluble glycoprotein from red cell membranes markedly inhibited the H PHAP induced lymphocyte stimulation but inhibited the L PHAP induced stimulation slightly or not at all The glycoprotein inhibited the H PHAP induced agglutination of red cells and leucocytes platelets, but did not inhibit the L PHAP induced agglutination of leucocytes-platelets

Various preparations of phytohaemagglutinin (PHA) from the red kidney bean (*Phaseolus vulgaris*) are glycoproteins with molecular weights of 80,000 to 130 000 PHA has been separated into two components both of which have leucoagglutinating and lymphocyte-stimulating activities but which differ in that only one of the components has erythro-agglutinating activity (*Adelson et al* 1965 *Weber et al* 1967) The erythro agglutinating and lymphocyte-stimulating activities of this component seem to be due to different parts of the same molecule (*Rigas & Johnson* 1964 *Rigas & Head* 1969 *Borjeson* 1971)

A complex of PHA and RBC membranes induces lymphocyte stimulation more effectively than does PHA in the absence of RBC membranes (*Tärnvik* 1971 a) The formation of PHA RBC membrane complexes can be inhibited by soluble glycoproteins or glyco peptides from RBC membranes (*Kornfeld & Kornfeld* 1969 1970) In the present study a glycoprotein was prepared from RBC membranes The effect of this glycoprotein on the agglutinating and lymphocyte stimulating activities of erythro-agglutinating and non erythro-agglutinating PHA is reported

MATERIALS

The ion exchange resins AG 50W X4 (200-400 mesh) and AG 1 X8 200-400 mesh) were obtained from Bio Rad Laboratories Richmond California and Amberlite CG 400 type I (100-200 mesh) from British Drug House Ltd Poole England Silica gel plates (SL 254 20×20 cm) for thin-layer chromatography were obtained from Antec AG Birsfelden Switzerland For column

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chromatography SP Sephadex C 50, Sephadex G 100, Sepharose 4 B and blue dextran 2000 were obtained from Pharmacia, Uppsala, Sweden

Bacto phytohaemagglutinin P (PHAP) was obtained from Difco Laboratories, Detroit, Michigan. Parker TCM 199 was from Flow Laboratories, Irvine Ayrshire, Scotland, benzylpenicillin from KABI AB, Stockholm, Sweden, and streptomycin sulphate from Glaxo Laboratories, Greenford England. The scintillation liquid, Instagel™ was from Packard Instrument Co., Inc., Downers Grove, Illinois, and [methyl ^{3}H] thymidine (specific activity 6.7 Ci per mmole) from New England Nuclear Corp., Boston, Massachusetts. Bovine serum albumin was from Armour Pharmaceutical Co., Chicago, Illinois. All chemicals used were of reagent grade quality.

METHODS

Phytohaemagglutinin. PHAP was chromatographed on SP Sephadex C 50 as described by *Weber et al.* (1967).

Fractions obtained by elution at pH 6.0 were pooled as were those obtained at pH 8.0. Their protein content was determined according to *Lowry et al.* (1951), using weighed amounts of PHAP as standard. Both these pools had leuco agglutinating and lymphocyte stimulating activities. The pH 8.0 pool (H PHAP) but not the pH 6.0 pool (L PHAP), had erythro agglutinating activity (*Weber et al.* 1967, *Tarnvik* 1971 b). The agglutinating properties of the pools were similar to those of the H PHAP and L PHAP described by *Allen et al.* (1969).

Preparation of glycoprotein from RBC membranes. Out dated bank blood was treated as described by *Kornfeld & Kornfeld* (1970). Blood was centrifuged to remove plasma. Cells were washed three times with 3 volumes of 0.9 per cent (w/v) NaCl–0.01 M $NaHCO_3$ and were then lysed with 9 volumes of water. The RBC membranes were sedimented at 12 000 × g for 12 min. The packed ghosts were then washed eight times with 7 volumes of 0.01 M Tris–0.001 M EDTA pH 7.4 to remove residual haemoglobin. Packed membranes were mixed with 9 volumes of chloroform methanol (2:1) and the mixture was vigorously shaken for 30 min. The aqueous layer was concentrated in a rotary evaporator and dialysed against 0.9 per cent (w/v) NaCl. The dialysed material was centrifuged at 2 800 × g for 1 hour to remove insoluble products.

Analytic procedures. The total neutral sugars were determined on the intact glycoprotein with the anthrone reagent (*Scott & Melvin* 1953), and with the phenol sulphuric acid method (*Dubois et al.* 1956). Galactose was used as a standard.

The hexosamines were determined after hydrolysis of 2 mg glycoprotein in 1 ml of 3 N HCl at 100° C for 4, 7, and 10 hours in evacuated sealed tubes. After removal of the HCl by lyophilization, the material was dissolved in water and transferred to a 1.5 ml column of AG 50W–X4 (H^+). The column was washed with 10 ml of water. The hexosamines were eluted with 5 ml of 2 N HCl (*Boas* 1953) and an aliquot was dried and analysed according to *Davidson* (1966). N acetylglucosamine was used as a standard. The recovery was about 90 per cent.

Sialic acid was released from 4 mg glycoprotein by hydrolysis in 5 ml of 0.1 N H_2SO_4 at 80° C for 1 hour in screw capped test tubes. The same results were obtained when hydrolysis was performed in 0.05 N H_2SO_4 at 90° for 1 hour. The material was transferred to a 5 ml column of Amberlite CG-400 (acetate). The column was washed with 10 ml of water, and sialic acid was eluted with 8 ml of 1 M sodium acetate buffer, pH 4.6 (*Svennerholm* 1958). The sialic acid was determined by the thiobarbituric acid method (*Warren* 1959) and by the resorcinol method (*Svennerholm* 1958). The recovery was about 95 per cent.

Methylpentose was determined on the whole glycoprotein with the method of *Dische & Shettles* (1948). Protein and hexose blanks were included. Fucose was used as a standard.

Total nitrogen was determined according to *Lang* (1958). The Nessler Folin reagent was prepared according to *Koch & McMeekin* (1924). Bovine serum albumin was used as a standard. The nitrogen content of this standard solution was confirmed with an ammonium sulphate standard.

Protein was determined with the biuret reaction (*Westley & Lambeth* 1960) and with the Folin phenol reaction (*Lowry et al.* 1951). Bovine serum albumin was used as a standard.

For the identification of neutral sugars about 25 mg glycoprotein was hydrolysed in 25 ml of 0.025 N H_2SO_4 at 100°C for 18 hours in volumetric flasks. The hydrolysate was passed through a 25 ml column of AG 50W X4 (H^+) and a 25 ml column of AG 1–X8 (formate) for the removal of sulphate ions, charged sugars and amino acids (*Spiro* 1966). The effluent and wash (200 ml) from the columns were evaporated to dryness in a rotary evaporator at 37° C. The material was dissolved in water and the content of reducing sugars was determined with the method of *Somogyi* (1945).

For thin layer chromatography the following systems were used: silica gel plates developed three times in methyl acetate isopropanol water (18:1:1) according to *Gal* (1968); silica gel plates saturated with 0.2 M sodium dihydrogen phosphate and developed in *n* butanol acetone water (4:5:1) according to *Hotta & Kurokawa* (1968). The plates were developed in sandwich chambers. After development the spots were detected by spraying

the plates with a silver nitrate reagent (*Gal* 1968) or a naphthoresorcinol-sulphuric acid reagent (*Lato et al* 1968)

To estimate the dry weight of the glycoprotein, a 10 ml sample of the glycoprotein in 0 9 per cent (w/v) NaCl was lyophilized for 2 days in the presence of phosphopentoxide The same volume of 0 9 per cent (w/v) NaCl was also lyophilized, and its dry weight was subtracted from that of the glycoprotein sample

Gel chromatography Sephadex G-100 (2 5 × 93 cm) and Sepharose 4 B (1 9 × 87 cm) columns were used The columns were packed and eluted with 0 9 per cent (w/v) NaCl Control chromatograms were run with blue dextran as a marker of the 'void' volume and glucose as a marker of the 'inner' volume The flow rate was 17 ml per hour and the effluent was collected in fractions of 10 ml volume In each fraction the absorbancy at 220 nm was read (*Waddell & Hill* 1956) and the content of total neutral sugars (*Dubois et al* 1956) was determined

Agglutination Washed human RBC were diluted with 0 9 per cent (w/v) NaCl to $7 5 \times 10^7$ cells per ml In order to obtain leucocytes platelets buffy coat of human blood was centrifuged in silica polyvinylpyrrolidone (*Pertoft et al* 1968, *Tarnvik* 1970 a) and cells from the mononuclear band were collected They were diluted in 0 9 per cent (w/v) NaCl to 3×10^7 leucocytes per ml In the agglutination tests 30 µl of RBC or leucocyte platelet suspension, 30 µl of L PHAP or H PHAP, and 30 µl of various dilutions of the glycoprotein in 0 9 per cent (w/v) NaCl were mixed The mixture was applied to glass slides, incubated at 37° C for 1 hour in humidified air, and was then examined microscopically The concentration of L-PHAP or H PHAP was 25 µg of protein per ml of mixture In the absence of glycoprotein this concentration of L-PHAP agglutinated leucocytes platelets and this concentration of H PHAP agglutinated leucocytes-platelets and RBC (*Tarnvik* 1971 b)

Culture medium The culture medium consisted of 80 per cent Parker TCM 199 and 20 per cent pooled inactivated (56° C 30 min) human serum The culture medium also contained 150 units of benzylpenicillin and 150 µg of streptomycin sulphate per ml

Lymphocyte cultures Venous blood was obtained from healthy blood donors Lymphocytes were isolated from 300 ml of blood by filtration through a nylon fibre column, followed by differential centrifugation in colloidal silica polyvinylpyrrolidone (*Tarnvik* 1970 a) The lymphocytes were suspended in culture medium to the final density of $0 7 \times 10^6$ cells per ml The suspension was distributed in cultures of 1 5 ml volumes in test tubes.

RBC were obtained from the same sample as used in the preparation of lymphocytes Blood was filtered through a nylon fibre column It was centrifuged and plasma was discarded Various numbers of RBC were added to various dilutions of glycoprotein, and 0 2 to 0 4 ml of this mixture was added per lymphocyte culture Fifty µl of L PHAP or H PHAP were added to each culture to the final concentration of 4 µg protein per ml of culture

The test tubes were loosely capped with aluminum foil and incubated for 62 hours at 37° C in humidified air supplemented with 5 per cent (v/v) CO_2 Fourteen hours before the end of the culture period, 1 5 µCi of ^{3}H thymidine in 30 µl of deionized water was added to each tube The radioactivity of ^{3}H-thymidine incorporated into DNA was measured (*Börjeson et al* 1966 *Tärnvik* 1970 b) Instagel™ was used as scintillation liquid

RESULTS

The material extracted from the RBC membranes had the characteristics of a glycoprotein (Table 1) The glycoprotein had a high content of sialic acid and a low content of fucose Thin-layer chromatography of the neutral sugars (15 µg) showed one heavy, slightly tailing, spot with an RF value corresponding to that of galactose, and two faint distinct spots corresponding to fucose and mannose The composition of the glycoprotein is similar to that of glycoproteins previously isolated from RBC membranes (cf

TABLE 1 *Analyses of Glycoprotein from RBC Membranes*

Component	Per cent of dry weight
Total nitrogen	9
Protein (as bovine serum albumin	
by the biuret method)	40
(as bovine serum albumin by the	
Folin phenol method)	36
Neutral sugars as galactose by the	
anthrone method	14
(as galactose by the phenol sulphuric	
acid method	13
Hexosamine (as n acetylglucosamine)	10
Sialic acid	
(by the thiobarbituric acid method)	15
(by the resorcinol method)	15
Methylpentose (as fucose	1

Winzler 1969) The glycoprotein appeared immediately after the 'void volume in the Sephadex G-100 column Four per cent (as measured by absorbancy at 220 nm) of the material applied to the column appeared as a distinct peak at the 'inner' volume of the column When the glycoprotein following the 'void' volume in the Sephadex G 100 column was applied to the Sepharose 4 B column, it appeared as a single peak (Fig 1)

Lymphocytes were incubated in the presence of L-PHAP or H PHAP and 3×10^6 RBC per ml of culture In the presence of L PHAP and glycoprotein, the incorporation of ^{3}H thymidine into DNA was slightly lower or equal to the incorporation obtained in the absence of glycoprotein In the presence of H PHAP and glycoprotein, on the other hand, the incorporation was markedly lower than that obtained in the abscence of glyco protein A similar effect was obtained with three different preparations of glycoprotein (Fig 2)

Lymphocytes were incubated in the presence of H PHAP and various number of RBC The incorporation of ^{3}H thymidine into DNA decreased with increasing amounts of glycoprotein added, irrespective of the number of RBC present (Fig 3)

The effect of the glycoprotein on the agglu tination induced by L-PHAP and H PHAP was studied L PHAP agglutinated leuco- cytes platelets but did not agglutinate RBC, the glycoprotein did not influence the L PHAP-induced agglutination of leucocytes platelets H-PHAP agglutinated leucocytes platelets as well as RBC both these agglutinations were inhibited by the presence of the glycoprotein (Table 2)

DISCUSSION

Lymphocytes purified from other cells respond to a low extent to PHA (*Levis* & *Robbins* 1970, *Tärnvik* 1970 b) The lympho cyte response to erythroagglutinating PHA (H PHAP) is increased by addition of RBC or RBC membranes to the culture (*Yachnin et al* 1969, *Johnson* & *Kirkpatrick* 1970, *Tärnvik* 1970 b, 1971 a, b) H-PHAP binds to RBC membranes (*Barkhan* & *Ballas* 1963 *Kornfeld* & *Kornfeld* 1969, 1970) A com plex of PHA and RBC membranes induces lymphocyte stimulation more effectively than does PHA in the absence of other cells or cell membranes (*Tärnvik* 1971 a) In the present study a glycoprotein from RBC mem branes was shown to inhibit the lymphocyte stimulation induced by H-PHAP, whereas the stimulation induced by non erythro-agglutinating PHA (L-PHAP) was virtually unaffected by the glycoprotein

One possible explanation of this result is that the glycoprotein interferes with the formation of PHA-RBC membrane com plexes and inhibits the lymphocyte stimula tion This explanation is not excluded by the fact that the glycoprotein inhibited the H PHAP induced lymphocyte stimulation even when no RBC had been added In the latter situation the glycoprotein might have inter fered with the formation of complexes between the H-PHAP and the few RBC or other cells present in the purified lymphocyte preparations Another possible explanation is that the interaction between H PHAP and glycoprotein changes the structure of H PHAP to such an extent that H PHAP can not combine with its receptor on the lympho cyte This might be due to a conformational change of the H PHAP molecule or to steric hindrance of that part of H PHAP which reacts with the lymphocyte A third explana tion is that the structure of the glycoprotein and the PHA receptor on the lymphocyte are so similar that they compete for the PHA molecules This explanation seems to be plausible only if it is assumed that the recep- tor on the lymphocyte involved in the H PHAP induced stimulation is different from the receptor involved in the L PHAP induced stimulation Otherwise, the glycoprotein would have inhibited the L-PHAP induced stimulation as well

Kornfeld & *Kornfeld* (1969, 1970) have isolated a glycopeptide of the RBC mem- brane which inhibited the erythro-agglutina ting activity and the lymphocyte stimulating

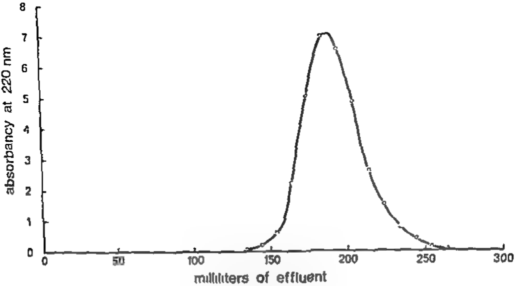

Fig 1 Chromatography of glycoprotein from RBC membranes (6 mg in 4 ml) on 1 9 × 87 cm Sepharose 4B column with 0 9 per cent (w/v) NaCl solution Flow rate 17 ml per hour, 10 ml fractions The peak of high molecular weight blue dextran 2000 was at 110 ml and of glucose at 280 ml

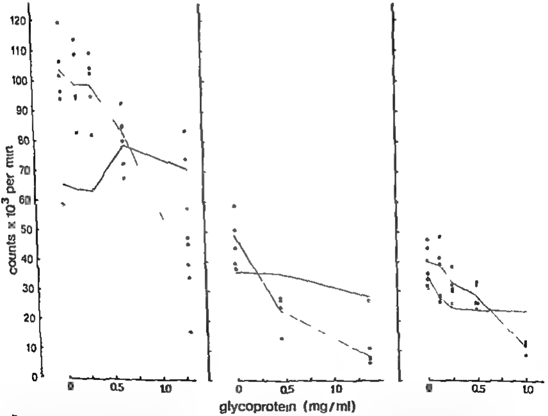

Fig 2 The effect of glycoprotein from RBC membranes on the lymphocyte response to H PHAP (●) and L-PHAP (O) The incorporation of ^3H thymidine into DNA was measured Three experiments with different batches of glycoprotein and with lymphocytes from different donors are shown

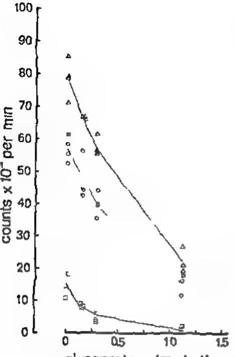

Fig 3 The effect of glycoprotein from RBC membranes on the lymphocyte response to H PHAP in the absence of RBC (□) and in the presence of 3×10^{6} (O) or 10^{7} (△) RBC per ml of culture The incorporation of ^{3}H thymidine into DNA was measured

activity of PHA It was suggested that the results demonstrated a similarity between the PHA receptor on RBC and the PHA receptor on lymphocytes involved in stimulation However this glycopeptide might also inhibit the formation of lymphocyte stimulating PHA RBC membrane complexes

L PHAP agglutinates leucocytes platelets but not RBC whereas H PHAP agglutinates both these cell preparations There are alternative explanations of this difference between L-PHAP and H PHAP One explanation is that L PHAP and H PHAP contain a common part capable of combining with leucocytes platelets and that H PHAP in addition contains another part capable of combining with RBC This explanation is not compatible with the present results The glycoprotein from the RBC membranes inhibited H PHAP induced but not L PHAP induced leucocyte platelet agglutination Thus it is unlikely that the parts of H PHAP and L PHAP combining with leucocytes platelets are similar

An explanation that is compatible with the present results is that leucocytes platelets contain two different receptors one combining with L PHAP and the other with H PHAP RBC on the other hand contain only one kind of receptor, combining with H PHAP This receptor is similar to that receptor of leucocytes platelets which combines with H PHAP

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TABLE 2 *Inhibition of Blood Cell Agglutination by Glycoprotein from RBC Membranes*
Thirty µl of RBC or leucocyte platelet suspension 30 µl of L PHAP or H PHAP solution and 30 µl of glycoprotein solution were mixed The RBC and leucocyte platelet suspensions contained 7 5 × 10 RBC and 3 × 10 leucocytes per ml respectively and the PHA solution contained 75 µg of protein per ml

PHA	Cells	Glycoprotein (µg)					Control
		43 8	0 68	0 34	0 17	0 09	
L PHAP	Leucocytes platelets	+ +	+ +	+ +	+ +	+ +	+ +
H PHAP	Leucocytes platelets	—		+	+ +	+ +	+ +
H PHAP	RBC	—	—	—	+	+ +	+ +

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THE LYSIS OF HUMAN DIPLOID FIBROBLASTS WITH BORATE BUFFER

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In the course of other investigations with human diploid fibroblasts (1 2) it was observed that borate buffer caused the cells to swell and lyse, leaving the nuclei intact The outer cell membrane appeared to be completely dissolved Some preliminary investigations on this phenomenon are described here

Human embryonic diploid fibroblasts obtained from lung tissue had the growth and chromosome characteristics expected of such cells (3 4) The culture conditions for these cells were similar to those reported earlier (5, 6 7)

A cloned line of HeLa cells grown in suspension culture was used for comparative experiments

Eagle s medium (8) was used for both cell types supplemented with 10 per cent calf serum 4 mM glutamine 1mM Na pyruvate 100 I E /ml and 100 μg/ml penicillin and streptomycin respectively

Stock solutions of 0 2 M boric acid and 0 2 M Na borate were mixed to obtain various pH s After dilution the final pH was determined

Equal aliquots of cells suspended with 0 25 per cent trypsin were centrifuged the supernatant decanted thoroughly the cells resuspended in 2 5 ml cold borate in an ice water bath and then rapidly brought to the appropriate temperature Samples were counted in a haemocytometer after 15 minutes incubation which was found to be the optimum time Only nuclei completely free of surrounding debris were scored The proportion of cell lysis was estimated by comparing the number of free nuclei in the test sample with the number of cells in the control (borate buffer + 0 8 per cent NaCl) Most of the experiments were repeated at least 3 times, using two independent diploid cell strains

A sharp optimum of diploid cell lysis was obtained at 0 06 M borate (Fig 1) Only a small proportion of free HeLa nuclei was found without any significant optimum over the range of 0 03 M to 0 2 M For subsequent experiments 0 06 M borate was used for both cell types

The optimum pH for diploid cell lysis occurred over the range 7 6 to 7 8 HeLa showed no signifiant optimum Between 0° C to 20° C only a small amount of cell lysis occurred Above 20° C the lysis of diploid cells increased rapidly whereas the lysis of HeLa remained relatively constant The percentage of free diploid nuclei continued to increase up to 45° C the highest temperature tried without reaching an optimum Most of our experiments were done first at 37° C and then repeated at 40° C with similar results

The presence of salts interfered with this reaction Lysis of diploid cells in 0 06 M borate was completely inhibited by 0 2 per

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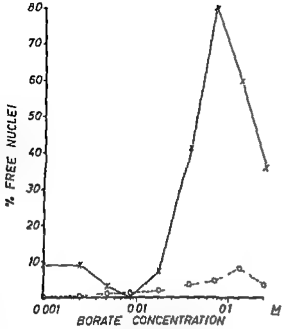

Fig 1 The effect of borate concentration on the liberation of free nuclei from diploid cells (×——×) and HeLa (o——o) The pH of the buffers was 7 4 The samples were counted after 15 minutes incubation at 37° C Similar results were obtained at 40° C

cent NaCl, 10^{-2} M $CaCl_2$ or 10^{-2} M $MgCl_2$

The nuclear membrane appeared to function as a semipermeable membrane when aliquots of free diploid cell nuclei were centrifuged and resuspended in solutions of different NaCl concentrations The morphology of the nuclei remained unchanged at concentrations less than or equal to 0 2 per cent NaCl At higher concentrations, the nuclei shrank to small, dense granules and at 3 to 5 per cent NaCl, the nuclear envelope was seen to be of normal size and shape, but the membrane appeared to be ruptured and the nuclear contents liberated When the nuclei were resuspended in KC1 solutions over the same concentration range, little or no shrinkage was observed Such osmotic effects have not been mentioned in descriptions of other methods by which to obtain free nuclei (9, 10, 11, 12) although *Laskowski* (13) stated that nuclear suspensions were more stable in KH_2PO_4

At present the mechanism of borate lysis is unknown

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GROWTH OF SIMIAN ADENOVIRUS SA7 DURING ARGININE STARVATION

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The effect of arginine starvation on the growth of simian adenovirus SA7 has been studied Omission of arginine from the medium inhibited production of infectious virus Extracts from virus infected cells maintained in arginine free medium were tested for immature virus particles Small amounts of virus activity found in these extracts seemed to be caused by the low levels of normal virus present, rather than by defective forms of virus

The presence of arginine in cell culture med[illegible]

absence of arginine reduces synthesis of viral DNA and capsid proteins but not to an extent comparable to the decrease of virus yield (*Rouse & Schlesinger* 1967, *Russell & Becker* 1968) Appearance of at least one viral component in the infected cells has been found to be very dependent on the presence of arginine, omission of arginine from the medium completely prevented formation of the "late" P antigen (*Russell & Becker* 1968) It was suggested that this late P antigen was similar to the maturation factor of some bacteriophages necessary for the components to assemble into infectious particles Defective viruses *i e* viral particles that are not infectious but do have some other viral activities, have been described in purified adenovirus populations (*Mak* 1971 *Rainbow & Mak* 1970), and can be produced by passing SV40 virus with high multiplicity inocula (*Uchida et al* 1968) In the present experiments, oncogenic simian adenovirus SA7 was grown under "nonpermissive" conditions caused by arginine starvation, and the extracts of these cells were studied to find out if viral intermediate forms were formed that would show some biological activity

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METHODS

Cells and viruses BSC 1 cells were used to grow and titrate SA7 virus (strain originally obtained from Dr *Herberling)* Cells were grown in Eagle's basal medium (Grand Island Biological Co) supplemented with 10 per cent calf serum and 5 per cent tryptose phosphate broth (Difco Laboratories) In the maintenance medium 2 5 per cent serum was used and L arginine (Schwarz Bioresearch Inc) was added to a concentration of 1mM Seed virus consisted of the low speed supernatant from infected cells broken by ultrasonication Plastic Petri dishes (35 mm diameter Falcon Plastics) were used for the plaque assay The agar overlay had the same serum and arginine concentrations as the maintenance medium. A second agar overlay with 0 005 per cent neutral red was added on the 7th day and the plaques were counted on the 12th to 14th day

Adenovirus type 2 (Ad2) was grown in KB cells and prepared for seed in the same way as SA7 virus. Ad2 was titrated in human embryonic kidney cells using the same procedure as for SA7 virus

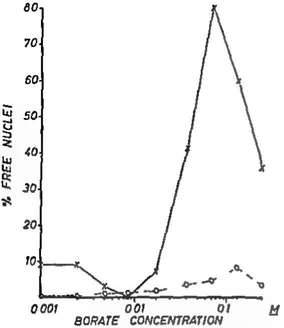

Fig 1 The effect of borate concentration on the liberation of free nuclei from diploid cells (×——×) and HeLa (o——o) The pH of the buffers was 7 4 The samples were counted after 15 minutes incubation at 37° C Similar results were obtained at 40° C

cent NaCl 10 " M $CaCl_2$ or 10 ² M $MgCl_2$

The nuclear membrane appeared to function as a semipermeable membrane when aliquots of free diploid cell nuclei were centrifuged and resuspended in solutions of different NaCl concentrations The morphology of the nuclei remained unchanged at concentrations less than or equal to 0 2 per cent NaCl At higher concentrations, the nuclei shrank to small, dense granules and at 3 to 5 per cent NaCl, the nuclear envelope was seen to be of normal size and shape, but the membrane appeared to be ruptured and the nuclear contents liberated When the nuclei were resuspended in KC1 solutions over the same concentration range, little or no shrinkage was observed Such osmotic effects have not been mentioned in descriptions of other methods by which to obtain free nuclei (9, 10, 11, 12) although *Laskowski* (13) stated that nuclear suspensions were more stable in KH_2PO_4

At present the mechanism of borate lysis is unknown

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GROWTH OF SIMIAN ADENOVIRUS SA7 DURING ARGININE STARVATION

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The effect of arginine starvation on the growth of simian adenovirus SA7 has been studied. Omission of arginine from the medium inhibited production of infectious virus. Extracts from virus infected cells maintained in arginine free medium were tested for immature virus particles. Small amounts of virus activity found in these extracts seemed to be caused by the low levels of normal virus present, rather than by defective forms of virus.

The presence of arginine in cell culture medium has been found essential for production of infectious human adenoviruses (*Rouse et al.* 1963, *Rouse & Schlesinger* 1967). The absence of arginine reduces synthesis of viral DNA and capsid proteins but not to an extent comparable to the decrease of virus yield (*Rouse & Schlesinger* 1967, *Russell & Becker* 1968). Appearance of at least one viral component in the infected cells has been found to be very dependent on the presence of arginine; omission of arginine from the medium completely prevented formation of the "late" P antigen (*Russell & Becker* 1968). It was suggested that this late P antigen was similar to the *maturation factor of some bacteriophages* necessary for the components to assemble into infectious particles. Defective viruses, i. e. viral particles that are not infectious but do have some *other* viral activities, have been described in purified adenovirus populations (*Mak* 1971 [illegible]

[illegible] present experiments, oncogenic simian adenovirus SA7 was grown under "nonpermissive" conditions caused by arginine starvation, and the extracts of these cells were studied to find out if viral intermediate forms were formed that would show some biological activity.

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METHODS

Cells and viruses. BSC-1 cells were used to grow and titrate SA7 virus (strain originally obtained from Dr. *Herberling*). Cells were grown in Eagle's basal medium (Grand Island Biological Co.) supplemented with 10 per cent calf serum and 5 per cent tryptose phosphate broth (Difco Laboratories). In the maintenance medium 2.5 per cent serum was used, and L-arginine (Schwarz Bioresearch Inc.) was added to a concentration of 1mM. Seed virus consisted of the low speed supernatant from infected cells broken by ultrasonication. Plastic Petri dishes (35 mm diameter, Falcon Plastics) were used for the plaque assay. The agar overlay had the same serum and arginine concentrations as the maintenance medium. A second agar overlay with 0.005 per cent neutral red was added on the 7th day and the plaques were counted on the 12th to 14th day.

Adenovirus type 2 (Ad2) was grown in KB cells and prepared for seed in the same way as SA7 virus. Ad2 was titrated in human embryonic kidney cells using the same procedure as for SA7 virus.

A hamster cell line (HDC–17) established in this laboratory (*Docherty et al* 1971) was used for the *transformation experiments It was grown in the* same way as the BSC–1 cells and was between the 21st and 23rd passage when used in these experiments Primary green monkey kidney (GMK) cells for complementation experiments were prepared by trypsinizat on and grown in the same way as the continuous lines

Growth of virus in the absence of arginine BSC–1 cell monolayers were infected with SA7 virus in a small volume at a multiplicity of one plaque forming unit (PFU) per cell After a one hour adsorption at room temperature the virus was washed off with tris buffered saline (0 025M tris 0 15M NaCl pH 7 4) Maintenance medium consisted of Eagle s medium lacking arginine with 2 *per cent dialysed calf serum or 0 1 per cent of* bovine serum albumin fraction V (Armour Pharmaceutical Co) To control cultures ($^{+}$arg), arginine was added to 1mM concentration For the growth studies virus was grown in one ounce bottles and harvested at the times indicated in Results For the rest of the experiments virus was harvested from $^{+}$arg cultures one to two days after infection and from arg cultures after 4 to 5 days At that time, cells were scraped off washed and resuspended in a small volume of tris buffered saline or in the same buffer containing 10mM EDTA and 1 5 000 of 2 mercaptoethanol Cells were broken by brief ultrasonication (Automatic Cleaner D 50 Branson Instruments Inc), and the extract used in further experiments

Density gradient centrifugations $^{+}$Arg and arg virus labelled with ^{3}H thymidine (20μC/ml specific activity 29C/mM Amersham/Searle) were prepared by freezing and thawing and sonicating infected cells in 0 01M tris buffer pH 7 5 containing 25mM EDTA and 1 5 000 of 2 mercaptoethanol Extracts were treated with 0 5 per cent BRIJ 58 (Atlas Chemical Industries Inc) and mixed with CsCl dissolved in the same buffer The average density of the gradient was calculated to be 1 40 gm/cm^{3} or 1 44 gm/cm^{3} Gradients were centrifuged in a Spinco L3 50 centrifuge with SW 50 1 rotor at 114 000g_{av} for 24 hrs Fractions were collected through the bottom of the tube on filter papers precipitated in 10 per cent trichloracetic acid washed in 95 per cent ethanol dried and counted in toluene liquifluor (New England Nuclear) scintillation liquid

RESULTS

Figure 1 shows the growth curve of SA7 virus in BSC 1 cells with and without arginine in the medium In $^{+}$arg cultures there was a complete cytopathic effect (CPE) in 24 hrs

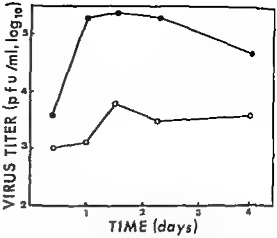

Fig 1 Growth of SA7 virus in BSC–1 cells with 1mM of arginine (●) or without arginine (○) in medium

after infection, in arg cultures little CPE was observed even 4 to 5 days after infection There was, however, some virus production also in arg cultures as shown by an increase of about 0 5 log in the titre At the time when $^{+}$arg cultures had the highest virus titre there was about 2 log difference in titres

If immature viral particles were present in arg cultures, they might lack infectivity because they are not able to adsorb on cells and/or because they are bound to membranous structures and are not released by sonication (*Mäntyjärvi & Russell 1969*) Therefore in the following experiments $^{+}$arg and arg viruses prepared as described in Methods were titrated in the presence of poly—L—ornithine (Sigma Chemical Co, molecular weight 90,000) or DEAE—dextran (Sigma Chemical Co, molecular weight 2× 10^{6}) A nonionic detergent, BRIJ 58, has been used to release replicating *E coli* deoxyribonucleoprotein particles from membranes (*Fuchs & Hanawalt 1970*) Therefore, in some of these experiments the cell extracts were treated with 0 1 or 0 2 per cent concentration of BRIJ 58 before adsorption Results of these tests are summarized in Table 1 The difference between $^{+}$arg and arg virus titres in ' normal ' titrations varied from 0 8 to 2 7 logs BRIJ pretreatment and adsorption with polyornithine or DEAE dextran had little effect on titres of either $^{+}$arg or arg virus In

TABLE 1 *Infectivity of SA7 Virus Grown with or without Arginine in Medium*

Virus	Pretreatment	Adsorption	Titre, p.f.u./ml, $\log_{10}$
+arg	–	–	6.4
	–	Polyornithine, 16μg/ml*	6.1
arg	–	–	3.7
	–	Polyornithine, 16μg/ml*	3.6
+arg	–	–	7.1
	BRIJ, 0.2%	Polyornithine, 30μg/ml*	6.4
arg	–	–	6.3
	BRIJ, 0.2%	Polyornithine, 30μg/ml*	5.4
+arg	–	–	6.4
	–	DEAE-dextran, 500μg/ml	6.4
	–	Polyornithine, 33μg/ml	6.5
+arg	–	–	[illegible]8
	–	DEAE-dextran, 500μg/ml	4.0
	–	Polyornithine, 33μg/ml	3.9
+arg	–	–	6.0
	–	Polyornithine, 33μg/ml	6.1
	BRIJ, 0.1%	Polyornithine, 33μg/ml	6.0
arg	–	–	3.8
	–	Polyornithine, 33μg/ml	3.8
	BRIJ, 0.1%	Polyornithine, 33μg/ml	3.9
+arg	–	–	7.9
	–	DEAE-dextran, 500μg/ml	6.8
	BRIJ, 0.1%	DEAE-dextran, 500μg/ml	6.9
arg	–	–	5.2
	–	DEAE-dextran, 500μg/ml	4.9
	BRIJ, 0.1%	DEAE-dextran 500μg/ml	4.6

* Cells were not washed after adsorption, i.e. polyornithine was present in the overlay

many experiments they caused decreases in titre

+Arg and arg viruses were then tested for transforming capacity in hamster cells They were adsorbed on monolayers of HDC 17 cells in the presence of DEAE-dextran (300 μg/ml) at room temperature for 30 min Cells were then washed, trypsinized and plated out in 60 mm plastic Petri dishes The total amounts of viruses used were 6×10^7 PFU for +arg virus and 1.3×10^6 PFU for arg virus The input multiplicities were 4 and 0.09 PFU/cell, respectively The cells were observed for 34 days and then stained, but no foci of morphologically transformed cells were found The experiment was repeated with another preparation of +arg and arg viruses with the same result To ascertain that HDC-17 cells were susceptible to transformation by SA7 virus, a control experiment was performed HDC-17 cells were infected with SA7 by shaking in suspension at room temperature for 3 hrs A multiplicity of 10 PFU/cell was used Cells were plated in 60 mm plastic Petri dishes The first transformed clones were found in these plates 8 days later After four weeks, the cells were stained and the foci counted Twelve foci were found giving a PFU/focus forming unit ratio of about 10^6 to 1

One of the biological activities of SA7 virus is to act as a helper for human adenoviruses in simian cells (Naegele & Rapp 1967) In the following experiment an attempt was made to find out if in arg virus preparations, there would be defective viruses which would

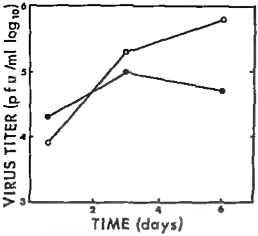

Fig 2 Growth of adenov rus type 2 in GMK cells alone (●) and with SA7 virus grown in the absence of arginine (○)

have this complementing capability Primary GMK cell monolayers in one ounce bottles were infected with Ad2 (5 PFU/cell, room temperature for 1 hr) Cells were washed and infected with ⁺arg and arg viruses in the presence of 500µg/ml of DEAE dextran at room temperature for 30 min Cells were washed again and maintenance medium containing SA7 antiserum was added Samples were harvested at 11 hrs and on the 3rd and 6th day Cells were broken by freezing and thawing and sonication and after adding SA7 antiserum titrated in human embryonic kidney cells for Ad2 Samples of the same cell extracts were titrated for SA7 virus in BSC 1 cells The results are shown in Fig 2

Arg virus seemed to be able to complement *Ad2 the titre of Ad2 on day 6 was 1 1 log* higher than the titre of Ad2 grown without helper In these samples there was however also an increase in SA7 virus titre from less than 10 PFU/ml at 11 hrs to $10^{3\ 6}$ PFU/ml on the third day and $10^{3\ 5}$ PFU/ml on the 6th day

The last experiments were designed to detect DNA containing viral particles which would have a different density from mature virus but which could not be identified by any biological activity monitored in the preceding experiments To do this ⁺arg and arg viruses were grown and labelled with ^{3}H thymidine as described in Methods ^{3}H thymidine was added 6 hrs (1st experiment) or 15 hrs (2nd experiment) after infection Cell extracts were prepared and centrifuged as described in Methods In the first experiment a peak was found at the density of mature virus (1 35gm/cm^3) in both ⁺arg and arg gradients, the peak of arg virus was about half of the peak of ⁺arg virus In both gradients there was a low wide peak at the bottom of the tube at a density of 1 5 gm/cm^3 Therefore, in the second experiment a denser gradient was used Virus was this time found in the top fraction, again some radioactivity was also present in arg gradient There was also some heavier material (density 1 6 gm/cm^3) in both gradients but the radioactivity profiles of both gradients were exactly the same

DISCUSSION

Replication of a number of DNA viruses including human adenoviruses has been shown to be sensitive to the absence of arginine from the medium (*Becker et al* 1967 *Goldblum et al* 1968 *Rouse et al* 1963, *Tankersley* 1964, *Winters & Consigli* 1971) In the present experiments this was found to be the case also with SA7 virus although the block was not complete as shown by a slight increase in the titre of infectious virus

The absence of arginine seems to preferentially affect the late events of adenovirus growth cycle (*Rouse & Schlesinger 1967 Russell & Becker 1968*) Therefore there could be formation and possibly accumulation of some DNA containing immature forms of virus in infected cells during arginine starvation DNA extracted from SA7 virus has been reported to be infectious (*Burnett & Harrington* 1968) However with methods used for detecting infectious DNA it was not possible to increase infectivity titres of arg cell extracts as compared to titres obtained with normal titrations The normal virus made in arg cells decreased the sensitivity of the assay About three times more defective par

ticles than normal virus would have to be present to be detected

Viral particles containing defective DNA, and therefore noninfectious may still have transforming capability *E g* by passing SV40 virus with high *multiplicity* inocula, it is possible to produce virus particles that are defective for infectivity but will induce T antigen in cell cultures and produce tumours in animals (*Uchida* & *Watanabe* 1968, *Uchida et al* 1968) Although the HCD 17 cells used in transformation experiments were shown to be susceptible to transformation by SA7 virus, no transformed foci were found in cultures infected with +arg and arg viruses The amount of +arg virus used should have been enough to induce foci but the adsorption time used was probably too short for normal virus The amount of normal virus in arg preparations was too small to give any foci and either there were no defective particles able to transform hamster cells or their number was too small to be detected by the methods used

The ability to enhance the replication of human adenoviruses in simian cells is a common property of SV40, PARA adenovirus and simian adenoviruses (*Rapp* 1971) PARA virus contains only about 75 per cent of the SV40 genome (*Kelly* & *Rose* 1971) suggesting that these viruses could carry complementing activity also in particles containing defective DNA In the complementation experiments with arg SA7 virus, SA7 antiserum was added after adsorption in order to avoid any effect on the hypothetical intermediate forms Antiserum therefore neutralized only the extracellular virus Normal virus of the arg preparation that had adsorbed replicated in GMK cells and the complementation observed after the 3rd day was probably caused by newly made SA7 virus rather than by any defective particles present in the original arg preparation

Two gradient centrifugation experiments to detect DNA containing particles with a characteristic density in arg virus preparations failed to do so Some denser DNA containing material was found in gradients (density 1 5 to 1 6 gm/cm³), but this was present in both +arg and arg preparations, and, therefore, was either of cellular origin or represented normal viral intermediate forms described in cell cultures infected with Ad5 (*Mantyjärvi* & *Russell* 1969) Thus, these results suggest that arginine deprivation blocks infectious virus production but does not lead to formation of mature, noninfectious virus particles

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RABBIT ANTI RAT LYMPHOCYTE SERUM: COMPARISON OF *IN VITRO* ACTIVITIES AND *IN VIVO* EFFECT OF THREE DIFFERENT TYPES OF ANTISERA

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In vitro lymphocytotoxic characteristics and the effect on skin allograft survival were studied Antisera raised with 3 injections showed more lymphocytotoxic activity in the IgM fraction than in the IgG fraction With increasing number of immunizations the IgM/IgG ratio as judged by the in vitro lymphocytotoxic test was reversed Antisera raised with 3 injections were much more effective than antisera raised with 8 injections in prolonging the survival of skin allografts The implications of these findings are discussed

There is as yet no defined procedure to make a potent immunosuppressive antilymphocyte serum (ALS) Considerable variation exists in the antigen used, the dose of antigen, the route of the administration and in the immunization schedule (14) The two pulse schedule of *Levey* & *Medawar* (21) has been adopted by many (8, 20) while more elaborate and irregular schedules have been used by others (1, 7, 9, 10)

Several publications indicate that antisera raised by few injections have greater immunosuppressive properties than do antisera raised by more prolonged immunization procedures (4, 6, 23 26) After a preliminary immunization course these animals have been bled repeatedly following subsequent booster immunizations

The immunosuppressive activity of ALS has been shown to reside in the IgG fraction of many antisera (2, 8, 10, 15, 20) *James* & *Medawar* (15) found no *in vivo* activity of the IgM fraction of their antisera

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It has previously been reported that considerable cytotoxic activity resided in the IgM fraction of antisera raised by a short immunization schedule (11) By increasing the number of injections of a standard dose, increasing cytotoxic activity towards lymphocytes was found in the IgG fraction

In the present study three different ALS using the same antigen dose, one raised by three twice weekly immunizations, one raised by three weekly immunizations and one raised by eight twice weekly immunizations have been investigated Different animals were used for raising the different antisera The *in-vitro* properties and *in vivo* immunosuppressive effect of these different antisera have been compared

MATERIALS AND METHODS

Preparation of ALS

Animals Albino rabbits used for raising the anti serum were all between 2 5 and 3 5 kg at the time of the immunization They were of both sexes They were fed commercial rabbit diet

Preparation of antigen Rat spleen cells were prepared as described previously (*11*). Spleens were cut into fragments and gently homogenized in a hand operated glass homogenizer (Quickfit BC 15/150) in 0.15 M NaCl. After filtration and washing the cells were finally suspended in Hanks' solution. Cells were counted in a Celloscope 401 (Ljungberg, Stockholm, Sweden) using a cetrimide solution (19). The relative number of viable spleen cells was determined using freshly prepared 0.2 per cent Trypan Blue in Hanks' solution. Viability counts showed 60–80 per cent viable cells. The cell suspensions were used for immunization within one hour of preparation.

Immunization procedure After a preimmune bleeding of 2–3 ml, three groups of rabbits were immunized. At each immunization 0.9–1.2 $\times 10^8$ viable spleen cells were given intravenously. One group received three injections over a period of one and a half week (referred to as "ultrashort"), the second group of rabbits were immunized once weekly for three weeks (referred to as "3 – pulse"), the third group of rabbits were immunized twice weekly for four weeks (referred to as "8 – pulse"). The sera were tested once or twice during the immunization procedure for cytotoxic activity. At each bleeding 5–6 ml of blood was collected from the ear vein. Ten days after the last immunization the rabbits were bled by heart puncture under Nembutal® anaesthesia. Serum was collected as previously described (11). Samples of antiserum from each rabbit were kept for in vitro studies. Serum from rabbits belonging to the same group of immunization schedule was then pooled, and stored at —20° C until used.

Absorption of anti red cell activity After inactivation by heating to 56°C for 30 min antisera were absorbed with rat red blood cells, washed thrice in 0.15 M NaCl. The absorption was carried out until the haemmagglutination titre was less than 16 using a 1 per cent solution of washed rat red blood cells in 0.15 M NaCl.

In Vitro Studies

The cytotoxic test The technique used for the preparation of the lymphocyte suspension and the procedure of the cytotoxic test have been described previously (*11*). Individual and pooled samples of normal rabbit serum and antisera were tested.

Mercaptoethanol treatment Reduction was carried out as described previously (11), using a final molarity of 0.1 M 2 mercaptoethanol for 2 hours, followed by alkylation with 0.02 M iodoacetamide ... 0.05 M phosphate buffer pH 7.2

In Vivo Studies

Schedule for administration of normal rabbit serum or ALS All sera had previously been absorbed with rat red blood cells and sterilized by passage through a Millipore filter 0.22 μ. Normal rabbit serum or ALS was given subcutaneously according to the following schedule: 2 ml daily from day —7 to day —1, 1 ml daily from day 0 (the day of the grafting) to day +7. Each rat thus received a total of 22 ml absorbed and filter sterilized serum. Each group of controls (untreated), "ultrashort", "3 pulse" and "8 pulse" consisted of 10 animals. The group receiving normal rabbit serum consisted of 5 animals.

Skin grafting technique Donors were male inbred Fischer rats, and inbred hooded BDE male rats were used as recipients (animals were obtained from Dyrlege Mollegaards Avlslaboratorium A/S, Ejby, Li Skensved, Denmark). Rats weighing between 200–250 grams were used for the transplant experiments. Full thickness square fitted grafts approximating 1.5 $\times$ 1.0 cm were sutured into a bed prepared on the back of the recipient. The grafts were thoroughly freed of panniculus carnosus as described by *Woodruff and Simpson* (25). The graft beds were subpannicular. Utmost care was taken to stop all bleeding before the graft was sutured into place, employing interrupted 4–0 silk. The graft was covered by a nonadhesive dressing (Adaptic, Johnson and Johnson), followed by two turnings of an elastic adhesive bandage. The bandage was left in place until day +8. From this day, grafts were inspected daily for viability. The day of rejection was taken to be the first day on which total destruction of the graft was evident (3, 29). It was not possible to perform the experiments "double blind".

RESULTS

In vitro Lymphocytotoxic Studies

None of the preimmune sera had a higher titre than 16 on testing for cytotoxic activity towards rat lymphocytes.

The results of the lymphocytotoxicity testing have been set out in Table 1. All three types of crude antisera showed cytotoxic activity, but the "ultrashort" ALS were less potent than the "3 pulse" or the "8 pulse" antisera, also comparing the content of activity after treatment with mercapoethanol. The difference between the "3 pulse" and the "8 pulse" as regards activity after reduction and alkylation by mercaptoethanol was only by one or two dilutions.

ABLE 1 *In Vitro lymphocytotoxic Titres of Individual and Pooled Differently Raised Rabbit Anti rat Lymphocyte Sera Comparison of Activity of Serum, Sterile Absorbed Serum, 2-Mercaptoethanol treated Serum and of IgM and IgG Fractions*

ype of serum	Crude serum	Sterile absorbed serum	ME* treated serum	IgM fraction	IgG fraction
Normal rabbit serum	16	8	4	<4	<4
"Ultrashort' ALS	512		64	4	<4
"Ultrashort' ALS	256		64	8	<4
"Ultrashort" ALS	256		32	8	4
"Ultrashort ALS	256		32	4	4
' Ultrashort' ALS pooled	256	256	32	8	4
' 3 pulse' ALS	2048		256	32	16
' 3 pulse" ALS	2048		256	32	16
3 pulse' ALS	1024		256	16	8
"3 pulse' ALS	2048		512	16	8
"3 pulse ALS pooled	2048	1024	128	16	8
8 pulse" ALS	4096		512	32	32
"8 pulse" ALS	2048		512	16	32
' 8 pulse" ALS	4096		1024	8	32
8 pulse" ALS	2048		1024	16	32
"8 pulse' ALS pooled	2048	2048	256	8	32

ME* – 2 mercaptoethanol

Separate testing of the respective IgM and IgG fractions showed only negligible activity in the IgG fraction and with small degree of activity in the IgM fraction of the "ultra short' antisera The "3 pulse" antisera all showed considerable activity in the IgM fraction, and all antisera that were tested showed lymphocytotoxic activity in the IgM fraction by one step more that in the IgG fraction On the contrary, with only one exception, the '8 pulse' antisera contained more activity in the IgG fraction, than in the IgM fraction

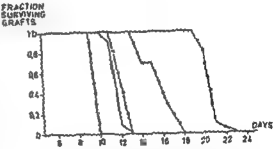

Fig 1 Survival curves for skin allografts from Fischer to hooded BDE rats Comparison of effect of different types of ALS ALS totalling 22 ml per rat given subcutaneously from day – 7 to day + 7 ——— untreated, —○—○— normal rabbit serum, —●—●— ultrashort ALS ■—■— 3 pulse ALS □—□— 8 pulse ALS

Skin Graft Survival

No wasting or haematuria was seen in any of the rats treated with the absorbed antisera

Untreated hooded BDE rats rejected a Fischer skin graft between the 11th and the 13th day Only 10 per cent of the grafts showed viability on day + 12 (Table 2, Fig 1) Normal rabbit serum did not prolong skin graft survival All grafts were rejected on day + 10 The pooled 'ultrashort' antisera did not increase the skin graft survival either, no viable graft was seen on day + 13

Both the pooled 3 pulse" and the pooled "8 pulse" antisera showed definite prolongation of skin graft survival There was, however, a striking difference between these two

TABLE 2 *Survival Data for Skin Allografts from Fischer to Hooded BDE Rats, Comparison of Effect of Three Different Types of Rabbit Anti Rat Lymphocyte Serum*

Type of treatment	No of animals	Range	Mean ± S D	Student T test
None	10	11–13	12 0±0 45	
Normal rabbit serum	5	10	10 0±0	p <0 001 versus control
'Ultrashort' ALS	10	12–13	12 4±0 49	p >0 05 versus control
'3-pulse ALS	10	20–23	21 1±0 77	p <0 001 versus control
"8-pulse' ALS	10	14–18	16 0±1 48	p <0 001 versus control p <0 001 versus 3 pulse

types of antisera, as treatment with the "3 pulse" antisera resulted in an average skin graft survival of 21 1 days, while similar treatment employing the '8 pulse" only resulted in an average survival of 15 3 days

The process of rejection of the skin allograft was equally rapid in the groups The normal looking skin graft became thick and red and further changed to hardened, non viable skin within 24–48 hours

DISCUSSION

The present study shows that antisera with different *in vitro* and *in vivo* properties can be obtained by varying the immunization procedure

Antisera produced by a short immunization schedule seemed to contain more lymphocytotoxic activity in the IgM than in the IgG fraction The cytotoxic activity of the IgM fraction of the "3 pulse antisera was somewhat greater than the IgM activity of the "8 pulse' antisera All the '8 pulse antisera contained most cytotoxic activity in the IgG fraction Comparing the "3 pulse" with the "8 pulse' antisera, the IgM/IgG ratio was reversed, as judged by the lymphocytotoxic activity *in vitro*

The "3 pulse' antisera definitely prolonged skin allograft survival, while "ultrashort' did not "8 pulse" antisera contained some immunosuppressive properties, but less than the "3 pulse" Normal rabbit serum did not show any immunosuppressive property

Many workers have shown that antisera raised with multiple injections have ability to delay the rejection and allografts (1, 5, 9, 25) *Shanfield et al* (25) found no change in the immunosuppressive activity comparing early and late bleedings during a prolonged immunization course Others have found actual decrease of immunosuppressive activity *in vivo* when comparing early and later bleedings of the same animals during a prolonged immunization course (4, 6, 23, 26) The present study confirms the latter findings Lack of any *in vivo* activity of the "ultrashort' antisera of the present study does not necessarily contradict this, as these antisera also had low *in vitro* activity

Previous workers noticing the decline in immunosuppressive potency with increasing number of immunizations (4, 6, 23, 26), have repeatedly bled their animals *James et al* (16) found that declining *in vitro* activity may be reversed by resting the serum raising animal before reboosting In this study different animals were used for raising "3 pulse' and "8 pulse" antisera thus avoiding rebleeding As this study confirms earlier findings it seems unlikely that repeated bleedings of the serum raising animal is responsible for the decline in the immunosuppressive activity of those antisera

Most studies of ALS have been done using crude antisera (4, 6, 7, 9, 17, 21, 23 25, 26) Few have tried to identify in *vitro* and *in vivo* activity of ALS in relation to the various classes of antibodies *James & Medawar* (15) described immunosuppressive activity residing only the IgG fraction This was recently confirmed by *Betel et al* (2) Many others have shown that the IgG fraction of their

antisera does contain immunosuppressive activity (8, 10, 20, 22).

The present study has shown difference between the "3 pulse" and the "8 pulse" antisera both with regard to the *in vitro* activity and of the ability to prolong the survival of skin allografts. Antisera with the best immunosuppressive properties contained most of the *in vitro* lymphocytotoxic activity in the IgM fraction, while antisera with strong *in vitro* activity of the IgG fraction were less potent *in vivo*. These observations suggest that there is some correlation between *in vitro* and *in vivo* activity of antisera. Previous studies have failed to show any definite correlation between *in vitro* cytotoxic and *in vivo* immunosuppressive properties (8, 14, 17, 18), although all immunosuppressive antisera are cytotoxic (18). Furthermore pepsin digestion of antilymphocytic antibodies eliminates both immunosuppressive properties and cytotoxic activity *in vitro* (1, 24).

The present results may be interpreted in one of the two ways. (I) if the immunosuppressive antibodies reside only in the IgG fraction of the ALS, one has to postulate that the IgG of the "3 pulse" antisera, though of less *in vitro* cytotoxic activity than the IgG of the "8 pulse" antisera, is somewhat better qualitatively speaking as regards *in vivo* immunosuppressive activity. Or (II) that other classes of antibodies at least partly may contain antibodies with immunosuppressive activity.

The less *in vivo* potency of the "8 pulse" antisera may be caused by a broadening of the antibody (IgG) specificity with increasing numbers of immunizations. Such a broadening of specificity may result in increased tendency to cross reactions with other cell types (21). Investigating the effect of ALS on macrophages in tissue culture monolayer it was found that "8 pulse" antisera had a much higher cytotoxic titre than did the "3 pulse" antisera (12). In vivo such broadening may lead to "attachment" of antilymphocytic antibody to other cells with subsequent loss of specific antilymphocyte effect.

James & Medawar (15) found no effect of the 19 S fraction *in vivo*. *Mandel & Asofsky* (22) found, however, immunosuppressive activity both of the IgG and the IgM fraction of their antisera on the graft versus host reaction. They stated that on a molar basis IgM antibodies might be as efficient as IgG antibodies in mediating suppression. The present study also suggests that the IgM fraction has immunosuppressive properties. This is further strengthened by the fact that antisera with *in vitro* activity in the IgM fraction do have some immunosuppressive effect, and that this effect is lost on treatment with 2-mercaptoethanol (13).

In conclusion the different properties of the present "3 pulse" and "8 pulse" antisera may be [illegible]
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longed immunizations.

The financial support of *Norsk Forening til Kreftens Bekjempelse*, *Landsforeningen* and *dr. A. Malthes fund* is gratefully acknowledged. The skillful technical assistance of *Anna Jerre* is also gratefully acknowledged.

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A POLYSACCHARIDE ANTIGEN OF AN ANAEROBIC ORAL FILAMENTOUS MICROORGANISM (*EUBACTERIUM SABURREUM*) CONTAINING HEPTOSE AND O-ACETYL AS MAIN CONSTITUENTS

2 *Physical Properties and Location in the Bacterial Cell*

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The antigen isolated from *Eubacterium saburreum*, strain L44, was found to be a neutral polysaccharide, which appeared in the electron microscope as a filamentous, partly aggregated material Immunoelectrophoresis gave one precipitation line, and a single peak was obtained by analytical ultracentrifugation of the purified antigen Experiments with ferritin labelled antibodies showed that the antigenic polysaccharide is located at the surface of the bacterial cell

A highly active polysaccharide antigen has been isolated from the anaerobic oral microorganism *Eubacterium saburreum* (4) Heptose and O acetyl accounted for approximately 90 per cent of the purified antigen In addition, all preparations contained small amounts of protein

The present paper deals with some physical properties of the isolated antigen, and with its location within the bacterial cell The serological properties of the antigen will be reported in another paper

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MATERIALS AND METHODS

The filamentous organism, strain L44, was cultivated in enriched nutrient broth (3) The polysaccharide antigen was extracted by digestion of acetone dried whole cells with trypsin and purified by passage through columns of Sephadex G 75 and DEAE-cellulose (4) Deacetylation of the lyophilized antigen was obtained by treatment with 0 01 N NaOH for 60 min at 56°C

Electrophoresis Cellulose acetate membrane (CAM) filter electrophoresis was carried out as described by *Kohn* (7), with a Shandon Electrophoresis Apparatus Model U77 Shandon Cellagram" cellulose acetate strips were used Samples were applied with a capillary tube or a Shandon Multi Applicator All runs were done with veronal buffer pH 8 6 ionic strength 0 06 for 60 min at an initial voltage of 25 V cm strip length Following electrophoresis the fixed strips were stained with amido black or the periodic acid Schiff stain Segments of dried CAM strips were also eluted with water and the eluates examined for serological activity by ring test precipitation Dying with Procion brilliant blue M R (Imperial Industries Ltd)

was done as described by *Dudman & Bishop* (1) The coloured polysaccharide was separated from inorganic salts and unreacted dye by gel filtration through a column of Sephadex G 25

Electrophoresis in 7 5 per cent polyacrylamide gel was carried out with a Shandon Disc Electrophoresis Apparatus Model SAE 2734 as described by *Hjertén et al* (2), using a continuous' buffer system A current of 3 mA to each running tube was applied and the electrophoresis continued until bromphenol blue had migrated through the polyacrylamide column

Immunoelectrophoresis was carried out with equipment type LKB 3276 B (LKB Produkter AB Stockholm Sweden) in 1 per cent agar gel (Difco Agar Noble) Electrophoresis was run for 1 hr at 250 V Following application of antiserum the plates were incubated at 4°C for 2 days

Gel filtration A 35 cm high column of Sephadex G 200 (AB Pharmacia Uppsala Sweden) of internal diameter 2 0 cm was stabilized and run at room temperature with 0 02 M phosphate buffer, pH 7 4 containing 0 001 M EDTA and 0 02 per cent sodium azide The void volume was determined by passing blue dextran, approximate molecular weight 2 000 000 ('Blue Dextran 2000 , Pharmacia Uppsala Sweden) through the column The *ion exchangers* Dowex 1 x8 and Dowex 50W were used in the Cl and H+ forms respectively

Analytical ultracentrifugation Velocity of sedimentation was examined in a Spinco Model E analytical ultracentrifuge at a rotor (Rotor Ad D) speed of 59 780 RPM The ultracentrifugations were performed at 20°C

Electron microscopy Single drops of a 0 05 per cent aqueous solution of the antigen were placed on carbon coated formwar membranes on copper grid and excess liquid carefully removed by touching filter paper to the edge of the grids The preparations were stained by floating the grids on a saturated solution of uranyl acetate in 50 per cent ethyl alcohol for 30 min followed by lead citrate for 5 min (9) and examined in a Philips EM 300 electron microscope operated at 80 kV Electron micrographs were recorded at magnification of 20 000 diameters

Immunoferritin technique Undiluted antiserum prepared against whole L44 microbes was absorbed with a heterologus *Eubacterium saburreum* strain in order to remove cross reacting antibodies 0 1 ml of the absorbed serum was mixed with 2 ml of a suspension in saline of washed L44 bacteria containing approximately 10^9 cells per ml Following incubation at room temperature for 30 min and three washings in saline the bacterial cells were suspended in 2 ml of saline and mixed with 0 1 ml of a ferritin IgG conjugate of goat anti rabbit gammaglobulin (Ferritin conjugate kit lot no 4117, Cappel Labs Inc Downington Pa) The mixture was allowed to stand at room temperature for 30 min and the treated cells washed as described Control experiments in which normal rabbit serum was substituted for antiserum L44 were included Prior to the experiments the reactivity of the commercial ferritin IgG conjugate was tested by immunoelectrophoresis against rabbit anti goat gamma globulin and rabbit anti horse ferritin

The antibody treated ferritin labelled bacterial cells and the cells treated with normal rabbit serum and the ferritin IgG conjugate were fixed in 1 per cent osmium tetroxide in Kellenberger buffer, embedded in epoxy resin (Durcupan Fluka AG, Buchs SG Switzerland), and sectioned for electron microscopy The thin sections were examined in the electron microscope without additional contrasting

EXPERIMENTS AND RESULTS

The purified and lyophilized polysaccharide antigen was a white powder readily soluble in water

In CAM electrophoresis experiments 0 8 µl samples of 0 1 to 1 per cent aqueous solutions of the polysaccharide antigen were applied to the cellulose acetate sheets by the multisample applicator No spots appeared following staining with amido black or the periodic acid Schiff stain However by eluting segments of dried and unstained filter strips serologically active material was obtained from the cathodic side of the application area In other experiments the polysaccharide antigen was dyed with Procion brilliant blue before CAM electrophoresis In addition to being coloured the polysaccharide antigen became deacetylated, as shown by agar precipitation against homologous antiserum (5) At the conditions used, the coloured polysaccharide migrated as a single diffuse spot approximately 0 5 cm towards the anode

In disc electrophoresis experiments the polysaccharide antigen did not enter the 7 5 per cent acrylamide gel

When immunoelectrophoresis was performed with veronal buffer, pH 8 6, ionic strength 0 06 the polysaccharide antigen and the deacetylated antigen moved towards the cathode (Fig 1) In order to examine whether this mobility was due to electro-osmosis, the immunoelectrophoresis experiments were re

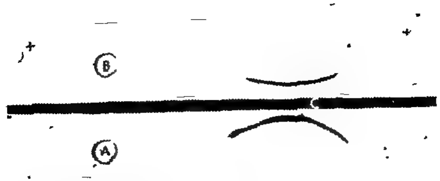

Fig 1 Immunoelectrophoresis of untreated (A) and deacetylated (B) polysaccharide antigen against antiserum L44 (C)

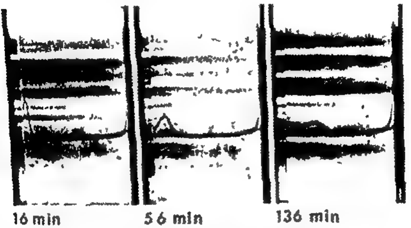

Fig 2 Schlieren pattern of the polysaccharide antigen Sample was 5 mg/ml in 0 05 M phosphate buffer, pH 6 8 Exposures were at 16, 56, and 136 min after reaching speed

peated with veronal buffers at pH 8 6, over an ionic strength range of 0 02 to 0 2 The movement towards the cathode of untreated and deacetylated polysaccharide varied inversely with the ionic strength, being maximal at ionic strength 0 02 In other experiments the influence of pH on the electrophoretic migration was examined at constant ionic strength 0 05, over a pH range of 3 8 to 8 6 Maximal movement towards the cathode was observed in phosphate buffer pH 7 4 The lowest migration rate was obtained with acetate buffer of pH 3 8 Dyed polysaccharide antigen and blue dextran, which were included in all experiments for comparison, moved similarly towards the cathode, but at a slower rate

The polysaccharide antigen was not retained on colums of Dowex-1 or Dowex-50

In separate gel filtration experiments, 0 5 ml of 0 25 per cent aqueous solutions of the polysaccharide antigen and the deacetylated antigen, respectively, were applied to the Sephadex G-200 column The eluted fractions were examined by double diffusion in agar against antiserum L44 The elution patterns were identical Both untreated and deacetylated antigen appeared in the void volume and in following fractions up to an effluent volume of $V_e = 3 V_0$

Ultracentrifugal analyses of untreated or deacetylated polysaccharide antigen were performed with samples dissolved at 5 mg per ml

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Analytical ultracentrifugation. Velocity of sedimentation was examined in a Spinco Model E analytical ultracentrifuge at a rotor (Rotor An D) speed of 59 780 RPM. The ultracentrifugations were performed at 20°C.

Electron microscopy. Single drops of a 0.05 per cent aqueous solution of the antigen were placed on carbon-coated formwar membranes on copper grid and excess liquid carefully removed by touching filter paper to the edge of the grids. The preparations were stained by floating the grids on a saturated solution of uranyl acetate in 50 per cent ethyl alcohol for 30 min followed by lead citrate for 5 min (9) and examined in a Philips EM 300 electron microscope operated at 80 kV. Electron micrographs were recorded at magnification of 20 000 diameters.

Immunoferritin technique. Undiluted antiserum prepared against whole L44 microbes was absorbed with a heterologous *Eubacterium saburreum* strain in order to remove cross-reacting antibodies. 0.1 ml of the absorbed serum was mixed with 2 ml of a suspension in saline of washed L44 bacteria containing approximately 10^9 cells per ml. Following incubation at room temperature for 30 min and three washings in saline the bacterial cells were suspended in 2 ml of saline and mixed with 0.1 ml of a ferritin IgG conjugate of goat anti rabbit gammaglobulin (Ferritin conjugate kit lot no 4117, Cappel Labs Inc Downington Pa.). The mixture was allowed to stand at room temperature for 30 min and the treated cells washed as described. Control experiments in which normal rabbit serum was substituted for antiserum L44 were included. Prior to the experiments the reactivity of the commercial ferritin IgG conjugate was tested by immunoelectrophoresis against rabbit anti goat gamma globulin and rabbit anti-horse ferritin.

The antibody treated ferritin labelled bacterial cells, and the cells treated with normal rabbit serum and the ferritin IgG conjugate were fixed in 1 per cent osmium tetroxide in Kellenberger buffer embedded in epoxy resin (Durcupan Fluka AG Buchs SG Switzerland), and sectioned for electron microscopy. The thin sections were examined in the electron microscope without additional contrasting.

EXPERIMENTS AND RESULTS

The purified and lyophilized polysaccharide antigen was a white powder readily soluble in water.

In CAM electrophoresis experiments 0.8 µl samples of 0.1 to 1 per cent aqueous solutions of the polysaccharide antigen were applied to the cellulose acetate sheets by the multisample applicator. No spots appeared following staining with amido black or the periodic acid Schiff stain. However by eluting segments of dried and unstained filter strips serologically active material was obtained from the cathodic side of the application area. In other experiments the polysaccharide antigen was dyed with Procion brilliant blue before CAM electrophoresis. In addition to being coloured the polysaccharide antigen became deacetylated as shown by agar precipitation against homologous antiserum (5). At the conditions used the coloured polysaccharide migrated as a single diffuse spot approximately 0.5 cm towards the anode.

In disc electrophoresis experiments the polysaccharide antigen did not enter the 7.5 per cent acrylamide gel.

When immunoelectrophoresis was performed with veronal buffer, pH 8.6 ionic strength 0.06 the polysaccharide antigen and the deacetylated antigen moved towards the cathode (Fig. 1). In order to examine whether this mobility was due to electro osmosis the immunoelectrophoresis experiments were re-

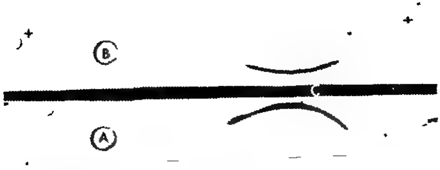

Fig 1. Immunoelectrophoresis of untreated (A) and deacetylated (B) polysaccharide antigen against antiserum L44 (C)

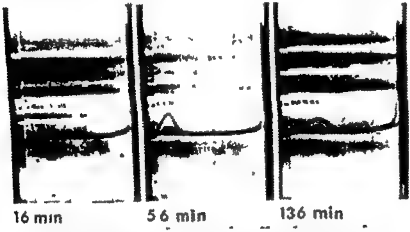

Fig 2 Schlieren pattern of the polysaccharide antigen Sample was 5 mg/ml in 0.05 M phosphate buffer pH 6.8 Exposures were at 16, 56, and 136 min after reaching speed

peated with veronal buffers at pH 8.6, over an ionic strength range of 0.02 to 0.2 The movement towards the cathode of untreated and deacetylated polysaccharide varied inversely with the ionic strength, being maximal at ionic strength 0.02 In other experiments the influence of pH on the electrophoretic migration was examined at constant ionic strength 0.05, over a pH range of 3.8 to 8.6 Maximal movement towards the cathode was observed in phosphate buffer pH 7.4 The lowest migration rate was obtained with acetate buffer of pH 3.8 Dyed polysaccharide antigen and blue dextran, which were included in all experiments for comparison, moved similarly towards the cathode, but at a slower rate

The polysaccharide antigen was not retained on colums of Dowex-1 or Dowex-50

In separate gel filtration experiments, 0.5 ml of 0.25 per cent aqueous solutions of the polysaccharide antigen and the deacetylated antigen, respectively, were applied to the Sephadex G 200 column The eluted fractions were examined by double diffusion in agar against antiserum L44 The elution patterns were identical Both untreated and deacetylated antigen appeared in the void volume and in following fractions up to an effluent volume of $V_e = 3\,V_0$

Ultracentrifugal analyses of untreated or deacetylated polysaccharide antigen were performed with samples dissolved at 5 mg per ml

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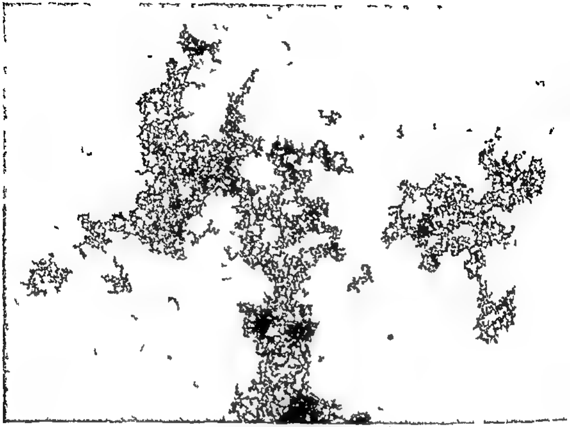

Fig 3 Polysaccharide antigen isolated from *Eubacterium saburreum* Electron micrograph × 100 000

in 0 05 M phosphate buffer pH 6 8 The antigens showed identical Schlieren patterns Both moved slowly as single peaks broadening during sedimentation (Fig 2) The uncorrected sidementation coefficients (S20) of untreated and deacetylated antigen at the concentration used were estimated to 3 86 and 4 0 respectively

In the electron microscope the positively stained polysaccharide appeared as a partly aggregated granular and threadlike material (Fig 3) The filaments had an approximate diameter of 30 A and varied in length The deacetylated antigen had the same appearance

The location of the polysaccharide antigen in the bacterial cell was investigated by means of the indirect ferritin conjugated antibody technique L44 bacteria treated with homologous absorbed rabbit antiserum and ferritin labelled antibodies to rabbit gamma globulin were surrounded by masses of ferritin particles in close contact with the outermost wall layer (Fig 4) Clearly resolved cross sections of bacteria showed no ferritin connected with the inner layers of the wall or the cytoplasm Clumps of ferritin particles were also seen between the cells unconnected with visible cell material or in loose contact with the bacteria With the exception of a few areas with ferritin tagged cells the bacteria in the control preparations were free of ferritin conjugated antibody

DISCUSSION

The polysaccharide antigen isolated from *Eubacterium saburreum*, strain L44 was seen in the electron microscope as a filamentous partly aggregated material The elution pattern on the Sephadex G 200 column is compatible with the threadlike appearance of the antigenic material and with the apparent heterogeneity with respect to molecular

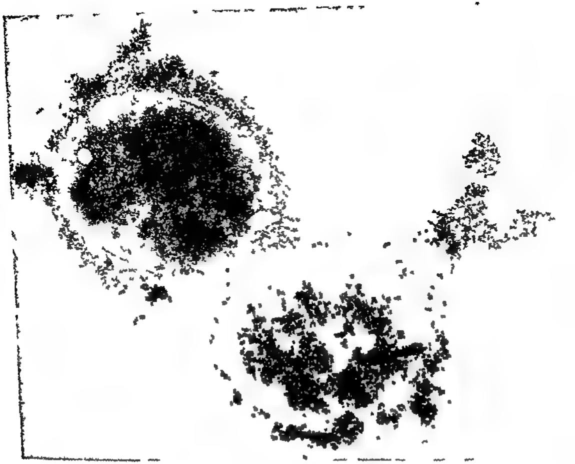

Fig 4 *Eubacterium saburreum* rabbit antiserum and ferritin labelled antibodies to rabbit gammaglobulin Electron den ritin part cles are located immediately adjacent to the periphery of the outer membrane of the cell wall Electron micrograph × 60 000

weight and degree of aggregation By analyt ical ultracentrifugation the polysaccharide sedimented slowly as a single fraction How ever polydisperse materials averaging about a mean sedimentation coefficient show but a single peak Polydispersity is also indicated by the broadening of the boundary observed during the sedimentation Because of the heterogeneity of the filamentous material it was found rather worthless to calculate its molecular weight

The passage of the isolated antigenic mat erial through columns of Dowex 1 (Cl) and Dowex 50 (H) suggets that the antigen is a neutral polysaccharide This is in agreement with the elution pattern on DEAE-cellulose (4) and with the results obtained by cellulose acetate membrane filter electrophoresis The migration of the antigen towards the cathode in the immunoelectrophoresis experiments varied with ionic strength and pH in the way characteristic of electroosmosis (8) The slo wer but similar movement of blue dextran may be explained by a higher molecular weight of the latter compound

The experiments performed did not dis close any contaminants in the antigen prepar ation The preparations treated with 0 01 N sodium hydroxide showed the same sediment ation velocity and electrophoretic migration as the untreated polysaccharide and were eluted similarly from the Sephadex G 200 column This shows that treatment with mild alkali does not confer major physico chemical

alterations on the polysaccharide antigen other than a deacetylation (4), characterized by a specific agar precipitation line (5)

Staining with the textile dye, Procion blue, which was carried out at alkaline conditions in the presence of sodium chloride (1), altered the electrophoretic migration of the polysaccharide antigen which also became deacetylated This shows that dyeing of polysaccharide antigens in the same way as textiles should not be done without reservation

Although the absorbed L44 antiserum was not monospecific for antibodies to the polysaccharide antigen, the experiments with ferritin labelled antibody strongly suggest that the antigen is located at the surface of the bacterial cell This suggestion is not invalidated by the finding of ferritin tagged cells in a few of the sections examined of bacteria treated with normal rabbit serum prior to treatment with ferritin conjugated antibody The presence of ferritin on the surface of these cells may be explained by unspecific adsorption of rabbit or ferritin labelled goat gamma globulin The polysaccharide antigen may be part of the outer cell wall membrane or identical with the granular material attaching to this membrane Both structures are removed by treating whole cells with trypsin (6)

The authors are indebted to Dr *Kjell Kleppe*, Department of Biochemistry, University of Bergen, for performing the analytical ultracentrifugation

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ISOLATION AND DESCRIPTION OF A HAEMOLYTIC SPECIES OF *NEISSERIA (N. ovis)* FROM CATTLE WITH INFECTIOUS KERATOCONJUNCTIVITIS

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In some cases of infectious keratoconjunctivitis in cattle haemolytic Gram-negative cocci were isolated in mixed culture together with *Moraxella bovis*. These Gram negative cocci were identified as *Neisseria ovis*. Differentiation from *Moraxella* may be difficult. An important differential characteristic is that *Moraxella* consists of short rods, whereas *Neisseria* are cocci. Unlike *M bovis*, *N ovis* is non-proteolytic. It reduces nitrate. The G + C content of the DNA of one of the strains was 43.9 per cent. Calves exposed to conjunctival infection with *N ovis* with and without previous ultraviolet irradiation did not develop any clinical symptoms.

From cases of infectious keratoconjunctivitis in Danish cattle *Moraxella* (M.) *bovis* has previously been isolated and described (*Pedersen* 1970). In some of the cases *M bovis* was found in mixed culture together with a haemolytic species of *Neisseria* (N.). As differentiation between the two genera may present certain difficulties a detailed description of the isolated *Neisseria* strains is given in the present communication.

MATERIAL

Haemolytic Gram negative cocci were isolated from 26 (52 per cent) of 50 animals showing clinical symptoms of infectious keratoconjunctivitis. They predominated in the bacterial flora of 6 animals. Simultaneous infection with haemolytic Gram negative cocci and *M bovis* was demonstrated in 24 animals (48 per cent). The present report deals with the results obtained in 15 strains of these Gram negative cocci. The isolated strains were compared with a type culture strain of *N ovis* (ATCC 17575) received from Dr *Reyn*, Statens Seruminstitut, Copenhagen.

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MEDIA AND METHODS

The media and methods were the same as those described in a preceding paper (*Pedersen* 1970) except that serum was not added to the media. Examinations for filtrable haemolysin were carried out in the medium suggested by *Smith* (1963). Calf erythrocytes were used for the tests.

Five calves 12–17 weeks old of the Red Danish Milkbreed (J. No. 38/71–72, 77/71–72, 78/71–72, 53/71–72, 82/71–72) were infected by conjunctival instillation of 0.1 ml of a 24 hours broth culture of *N ovis* (strain No. 3). All the calves originated from herds in which infectious keratoconjunctivitis according to information had never occurred. Two calves (J. No. 53/71–72 and 82/71–72) were only infected and not irradiated. Three calves (J. No.

38/71–72 77/71–72, 78/71 72) were irradiated twice by ultraviolet light on both eyes for 10 minutes at a distance of 60 cm with 24 hours interval (*Hughes et al* 1968) Immediately after the last irradiation the calves were infected on the one eye, while the other one served as control to the irradiation employed As source of ultraviolet light a commercial sunlamp was used The lamp emitted light with wavelengths 280 300 nm

RESULTS

Cell Morphology

The bacteria were non motile, non sporing Gram negative oval cocci which often occurred in diploid arrangement The adjacent sides were flattened, with the longitudinal axes at right angles to the axis connecting the two cells The diameter of the cells was about 0 5 1 0μ Swollen forms were also observed

Cultural Characteristics

Surface colonies on blood agar incubated under aerobic conditions at 37°C were 1 5 2 5 mm in diameter and had regular edges The colonies were surrounded by a 1 0 1 5 mm clear haemolytic zone They were low convex, with a smooth glistening surface The centre of the colonies was almost opaque while the periphery was more translucent The colour was greyish white After removal of the colonies shallow depressions of the agar surface were observed The depth of these were most pronounced centrally The walls of the depressions were smooth The consistency of the colonies was butyrous, and a homogeneous saline suspension was easily formed by rubbing a little colony material with a platinum loop After 48 hours incubation the diameter of the colonies was 3 4 mm At this stage the larger of the single colonies began to form daughter colonies which were evenly distributed over the surface and along the edges of the colonies

Colonies in blood agar pour plate incubated aerobically at 37°C for 24 hours were biconvex and surrounded by a 1 2 mm clear zone of haemolysis The colonies showed a tendency to grow through the surface

On inoculation in deep agar there was no growth in the butt after 48 hours incubation but growth was abundant on the surface and down to 1 mm below it

In stab cultures in semi solid agar there was growth along the entire stab and a coherent film of growth covered the entire surface

In meat peptone broth growth was good causing little turbidity There was a moderate greyish white sediment which formed a coherent viscous mass when shaken The sediment could be broken up by vigorous shaking resulting in a homogeneous suspension Growth occured on the surface and mainly along the walls of the tubes On the surface the culture formed a pellicle of varying thickness Growth occurred at 22°C The rate of growth was lower at 30°C than at 37°C

One strain (No 3) was examined for production of filtrable haemolysin It was not demonstrated Growth was poor under anaerobic conditions No pigment was formed on incubation at 22° and 37°C All strains grew well on non enriched media

Biochemical Reactions

All strains examined were catalase positive None of them fermented the following monosaccharides glucose arabinose fructose galactose mannose rhamnose and xylose neither did any of them split the disaccharides lactose maltose and saccharose

All strains reduced nitrate to nitrite None of them decomposed urea or produced hydrogen sulphide Indole was not produced there was no liquefaction of Loeffler s serum There was no growth on MacConkey agar Litmus milk remained unchanged

DNA Base Composition

DNA base composition was determined according to the procedure described by *Marmur* (1961) The percentage of guanine and cytosine (G + C) of the type culture strain was 44 2 and that of strain No 3 43 9

Based upon the examinations here reported including comparison with a type culture

strain of *N ovis* (ATCC 17575) the haemolytic Gram negative cocci are identified as *N ovis*

Antibiotic Sensitivity

The results of the antibiotic sensitivity tests are shown in Table 1 All strains examined were *sensitive* to penicillin chloramphenicol and neomycin They were *sensitive* to *fairly sensitive* to streptomycin and sulphonamide All strains examined were *fairly sensitive* to tetracycline

TABLE 1 Antibiotic Sensitivity of 15 Strains of *Neisseria ovis* Ranges of Inhibition Zone Diameters and Minimum Inhibition Concentrations (IC 50) *

Antibiotic	Inhibition zones mm	IC 50
Sulphonamide	26 34	5 0–1 1
Penicillin	32–4?	0 07–<0 01
Streptomycin	18 25	7 8–0 4
Chloramphenicol	26–32	1 60–0 38
Tetracycline	18 22	2 4 0 7
Neomycin	20–26	0 07 0 02

* IC 50 given as I U/ml for penicillin and as mcg/ml for other antibiotics

Pathogenicity

The calves only exposed to *conjunctival* infection with *N ovis* did not show any symptoms The calves which had been *irradiated* with ultraviolet light *showed slight* hyperaemia of the skin surrounding *the eyes and* slight serous lachrymation during the first few days after the irradiation No difference between infected and non infected eyes could be observed

Bacteriological examinations of swabs prior to infection revealed *that* none *of the calves* harboured *N ovis* on the conjunctival mucous membranes

Daily bacteriological examinations of conjunctival swabs *from* the calves after infection revealed that *N ovis* became established *in all* exposed eyes In three calves the organism was re isolated for 3 days after infection (J No 38/71 72 78/71 72 82/71 72) in one calf for 5 days (J No 77/71 72) and in another calf the bacterium was re isolated for 25 days after exposure

The pathogenicity to mice was examined by intravenous and intraperitoneal injection of a dense saline suspension of living bacteria (strain No 3) Groups of 5 mice each were given 0 1 ml intravenously 0 1 ml intraperitoneally and 0.5 ml intraperitoneally The mice which received 0 1 ml intravenously all died within an hour The mice given 0 1 ml intraperitoneally died within 18 hours after the injection The mice that were given 0 5 ml intraperitoneally died within 8 hours Haemoglobinuria was not observed Post mortem examination revealed no macroscopical changes other than the accumulation of a serous fluid in the thoracic cavity In all mice *N ovis* was re isolated from the heart blood and the pleural fluid

DISCUSSION

In 1960 *Lindquist* described a *Neisseria* species associated with infectious keratoconjunctivitis in Norwegian sheep and named it *N ovis Fairlie* (1966) isolated an organism from the eyes of Scotch calves and sheep irrespective of demonstrable disease The organism was considered as being a variant of *N ovis Fairly* (1966) isolated an organism first description from Denmark of a similar organism isolated from cases of keratoconjunctivitis in cattle Based on the examinations here reported it is identified as *N ovis*

In Australia *Wilcox* (1970) isolated three varieties of *Neisseria* from the bovine eye One was non haemolytic it was identified as *N catarrhalis* The other was haemolytic and was regarded as a haemolytic variant of *N catarrhalis* not as *N ovis* A third haemolytic variant was characterized by digestion of casein but was otherwise similar to *N catarrhalis*

N ovis appears to be closely related to *N caviae* their DNA base compositions are identical and the two species may possibly be variants of the same species (*Bøvre* 1967b) In conventional tests a haemolytic *N ovis* is practically indistinguishable from *N caviae*

(*Bövre* 1965) *N ovis* and *N caviae* are closely related to *N catarrhalis*, but they have different DNA base compositions (*Bövre* 1967b) The term "false" *Neisseria* ■ often used for *N catarrhalis*, *N caviae* and *N ovis* The three species differ from "true" *Neisseria* by having lower G + C contents of the DNA and ■ low genetic affinity to "true" *Neisseria* *Henriksen and Bövre* (1968) suggested the transfer of *N catarrhalis*, *N ovis* and *N caviae* to the genus *Moraxella*, but found the establishment of a new genus for the three species acceptable Recently, *Catlin* (1970) proposed that *N catarrhalis* be transferred to a new genus named *Branhamella*, but she did not consider the taxonomic classification of *N ovis* and *N caviae*

Studies based on transformation of resistance to streptomycin have revealed that *N ovis* and *M bovis* are closely related Genetic transformation of haemolytic properties from *M bovis* to a non haemolytic mutant of *N ovis* also indicates taxonomically important relations between *Moraxella* and *Neisseria* (*Bovre* 1967a)

On the examination of swabs from cattle with infectious keratoconjunctivitis differentiation of *N ovis* from *Moraxella* may prove difficult *M bovis* isolated from cattle has been described previously (*Pedersen* 1970) The strains of N *ovis* described in the present paper were catalase positive, whereas the strains of *M bovis* examined by the author were all catalase negative Unlike *M bovis*, *N ovis* is non-proteolytic As stated by *Henriksen* (1952), the cell morphology is of great value *in the differentiation of Neisseria from Moraxella*, the only significant difference between the two genera being that *Neisseria* consists only of cocci and *Moraxella* primarily of rods

The aetiological significance of *N ovis* in the development of infectious keratoconjunctivitis in cattle is not known *Fairlie* (1966) exposed two calves to conjunctival infection with *N ovis* but no inflammatory reactions were seen and the lachrymal fluid was sterile again after 24 hours In the present study no symptoms were observed after conjunctival infection even after ultraviolet irradiation of the eyes, ■ method described to enhance the effect of *M bovis* infection of the bovine eye (*Hughes et al* 1965) Hence, on the basis of these results there is little evidence of pathogenicity of *N ovis* In sheep *Spradbrow* (1971) recently reported that *N ovis* probably is ■ commensal organism well adapted to survival in the ovine conjunctival sac and not a primary pathogen

The significance of the simultaneous presence of *M bovis* and *N ovis* in cattle with infectious keratoconjunctivitis remains ■ be established Information on the occurrence of *N ovis* in normal cattle in Denmark ■ also lacking

Lindquist (1960) reported that *N ovis* caused haemoglobinuria in mice within two hours and death after 18 hours, when ■ saline suspension was injected intravenously *Fairlie* (1966) found that none of the strains isolated by him were fatal to mice even after injection of large doses The strain presently examined was fatal to mice but haemoglobinuria was not observed

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INHIBITION BY RABBIT ANTIBACTERIAL IgG OF THE BACTERICIDAL EFFECT ON SALMONELLA TYPHIMURIUM 395 MRO BY NORMAL CATTLE SERUM

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The component of rabbit anti Salmonella typhimurium 395 MRO responsible for specific inhibition of the bactericidal effect of normal cattle serum can be isolated by chromatographic fractionation The inhibitory fraction when examined by analytical ultracentrifugation, contained a single peak with sedimentation coefficient ($s_{o w}$) of 6 6 S Immunoelectrophoresis of this fraction revealed a single precipitation arc with anti-whole rabbit serum which gave a reaction of identity with the line developed by specific anti rabbit IgG serum The inhibitory effect of immune rabbit serum is concluded to be due to specific IgG antibodies directed against bacterial surface antigens

In the presence of rabbit antiserum the bactericidal effect of normal human or cattle serum on certain R-mutants from Salmonella typhimurium is extinguished (5,6) Since the antibactericidal activity of an antiserum was generally restricted to the homologous mutant or to mutants with similar or identical structure of the lipopolysaccharide (LPS) of the cell wall, antibodies were supposed to be responsible This communication is aimed at a characterization of the substance(s) involved in the antibactericidal activity of the immune rabbit serum

MATERIALS AND METHODS

Bacterial strain Salmonella typhimurium 395 MRO (chemotype Ra), used throughout this investigation was originally obtained from Dr T Holme, Stockholm It has been described in detail earlier (7, 9 11) The strain was kept at 4° C on agar slants and tested at intervals by phage pattern

Immunization Synthetically grown bacteria were used as immunogens Rabbits were injected intravenously according to two different immunization schemes

1) *The long time* scheme (sera labelled ROL) has been described in detail previously (5) In brief the bacteria were killed at 56° C for 1 hour and 0 2 mg (1 mg/ml dry weight) injected three times a week for nine weeks The rabbits were bled five days after the last injection

2) *The short time* scheme (sera labelled ROS) is a modification of the method described by Schlecht and Westphal (18) The bacteria were heated at 100° C for 1 hour and a suspension containing 0,75 mg/ml prepared Three doses (0 2 ml 0 5 ml and 1 ml) were injected at 5 day intervals The rabbits were bled five days after the last injection The blood was collected from the carotid artery and after clotting at room temperature for one hour it was placed at 4° C for another 1–2 hours and then centrifuged The sera were dispensed in small aliquots immediately frozen and kept at −20° C until used

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Cattle serum Blood from cattle was collected under clean conditions from the carotid artery at the slaughter house Serum was separated as above, checked for sterility and kept at —90° C until used

Fractionation of rabbit serums was always carried out at 5° C.

1) *DEAE cellulose* After overnight dialysis against the starting buffer (sodium phosphate, μ = 0 02, pH = 7 3) 2 ml of the serum sample was loaded on a column (0 9 × 30 cm) filled with DEAE-cellulose (Whatman) A linear salt gradient was obtained by connecting two identical beakers one filled with 150 ml of the starting buffer, the other filled with 150 ml 0 5 M NaCl A magnetic stirrer was placed in the first beaker to ensure a smooth gradient The flow rate was about 15 ml/hour and 3 ml fractions were collected Each separation was repeated once as a control

2) *Sephadex G 200* The serum sample was dialysed overnight against a triethanolamine buffer (2 24 g triethanolamine 7 89 g NaCl, 0 372 g EDTA and 1000 ml dist water pH adjusted to [illegible]

The flow rate was about 10 ml/hour and 3 ml fractions were collected Each separation was repeated once as a control

Protein determination The protein concentration was determined in a Beckman DB G spectrophotometer at 280 nm As reference for the non separated sera a standard human serum (Standard Human Serum Behringwerke AG) was used For the serum fractions a solution of human IgG (Kabi 1064) was employed

The bactericidal effect of normal cattle serum in the presence of antiserum or antiserum fractions was assayed in a way similar to the method described earlier (6) Twofold serial dilutions of the antisera (0 15 ml) were mixed with equal volumes of cattle serum (diluted 1 2) To each tube 0 3 ml of S typhimurium 395 MRO (approximately 4 × 10 bact./ml) was added and the tubes were incubated at 37° C for 1 hour The viable count was determined by spreading 0 1 ml volumes of ten fold-dilutions onto nutrient agar plates in duplicate Plates [illegible] mean [illegible] fraction [illegible] N_0 is [illegible] not sub[illegible] to serum and N the concentration of bacteria in the treated sample When the surviving fraction was plotted as serial dilutions of the rabbit antiserum [illegible] with its [illegible] The amo[illegible] conditions [illegible] 0 15 antibactericidal units (AU) because 0 15 ml volumes of rabbit serum were used The protein concentration (c) at S = 0 1 was calculated by interpolation and used to describe the antibactericidal potency (a) of the sample ($a = \frac{1}{c}$ AU/mg)

Passive haemagglutination A dimethyl sulfoxide (DMSO) extract (3) was treated with sodium hydroxide and used for coating sheep red cells (SRBC) (14) To serial two-fold dilutions of the serum in portions of 0 2 ml, the same volume of sensitized SRBC (0,5 per cent) was added After thorough mixing, the tubes were kept at room temperature for 18 hours and the sedimentation patterns observed Nonfractionated sera and the fractions with the highest haemagglutination titres were also tested with non sensitized SRBC No titre above 2 was recorded

Immunoelectrophoresis was used to check the immunological purity of the samples It was done according to Scheidegger (17) with Gelman equipment The electrophoresis was carried out with 1 5 per cent agarose (Difco), 0 05 M tris barbital buffer pH 8 8 and at 7V/cm for 100 min After the electrophoretic run one side basin was filled with goat anti rabbit IgG (Microbiological Associates Inc) and the other with donkey anti rabbit serum (Behringwerke AG) As a control the non separated rabbit antibactericidal serum was tested The slides were incubated at 37° C for 24 hours and for another 24 hours at 22° C

Ultracentrifugation The sedimentation coefficient of a purified fraction with high antibactericidal activity was determined in the analytical ultracentrifuge (kindly performed by Dr *H Pertoft*, Institute of Medical Chemistry, Uppsala, Sweden)

RESULTS

The two immunization procedures gave sera with different properties As a representative for the long time procedure the serum ROL–10 was chosen, and for the short time scheme, ROS–16 The haemagglutination titre of ROL–10 was 128, that of ROS–16 was 4096 The antibactericidal activities of the sera are depicted in Fig 1 In spite of a 32-fold higher haemagglutination titre of ROS–16, the antibactericidal activity of ROS–10 was higher, approximately 480 AU/ml, whereas that of ROS–16 was 58 AU/ml Other sera of these two immunization procedures had similar properties

After fractionation of ROL–10 on DEAE-cellulose, the antibactericidal activity was recovered in the first peak (Fig 2, tubes 3–7) which contains mainly IgG (8) The highest activity was noted in tubes 4 and 5 The

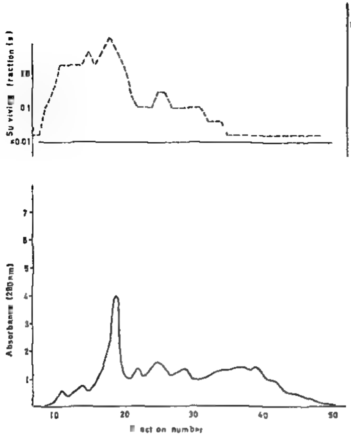

Fig 4 ROS–16 was fractionated on a Sephadex G 200 column No antibactericidal activity was recovered Surviving fraction (S) —— Haemagglutinating activity ----

of normal human or cattle serum on this mutant (5) The antibactericidal activity was generally specific for the immunizing R mutant or for mutants belonging to the same chemotype (6) These observations supported the idea that antibodies specific for the cell wall LPS which determines the chemotype, antagonized the bactericidal effect of normal sera They also excluded nonspecific anticomplementary factors as main causes in the extinction process The present experiments show that the antibactericidal activity has several characteristics of IgG, high titre after long time immunizations, localization in the IgG peak after fractionation on DEAE-cellulose (8), reaction of identity with anti-IgG at the characteristic location in immunoelectrophoresis and a sedimentation coefficient ($s_{20,w}$) of 6 6 S Earlier determinations of $s_{20,w}$ for rabbit IgG have given $s_{20,w}$ = 6 7 S (16) In contrast, the haemagglutinating activity showed several characteristic of IgM antibodies e g early appearance in the immunization period and localization in the first peak after fractionation on Sephadex G 200 which has repeatedly been found in similar systems (10)

Several investigations have demonstrated a complement dependent bactericidal effect of IgG antibodies, weaker, however, than that of IgM-antibodies (3, 15 16) The IgG-fraction from RO antiserum has, however never shown bactericidal activity, either with guinea pig or rabbit serum as complement source

It is known that at high concentrations of certain antisera the complement mediated bactericidal effect is reduced (13) Different explanations have been offered for this "prozone" phenomenon At the time of the discovery it was supposed that amboceptor and complement combined first and that the

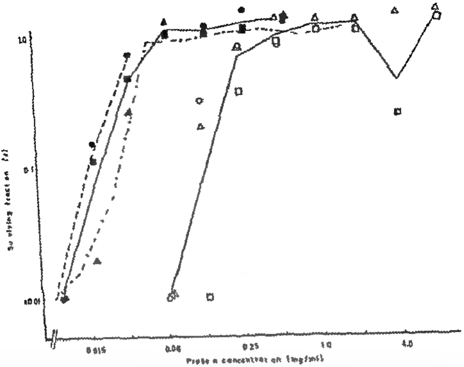

Fig 5 The antibactericidal activity of ROL–10 and of a sample obtained after two consecutive fractionations on DEAE-cellulose related to their protein concentrations The activities were determined on three separate days Solid lines are drawn through the average values of the three determinations The symbols represent individual tubes Open symbols = ROL–10, closed symbols = the antibactericidal sample The activities of fraction 3 and 4, obtained after one fractionation on DEAE-cellulose, are also recorded Fraction 3 – – Fraction 4 — — —

excess of antibody, uncombined with bacteria, deviated the complement activity from the bacteria Coombs (2) suggested complement deviation to soluble antigen-antibody complexes Competition for antigenic sites at the bacterial surface between complement fixing and non complement fixing antibodies has also been considered, but the latter have never been shown (see 12)

In experiments with S typhi O 901 using rabbit antiserum and guinea pig complement (3 12), both the antibody prozone phenomenon and the bactericidal effect were demonstrated with IgM and IgG fractions *Muschel et al* (12) found that in relation to the bactericidal antibody titre the IgG fraction showed higher prozone titre than the IgM fraction The IgM fraction, however, showed the highest absolute titre in both tests In addition to the prozone effect of the antibodies they noted a non-specific anticomplementary effect of serum particularly of heated serum

Daguillard and Edsall (3), working with antibodies eluted from S typhi at pH 3 5 found that, on a weight basis, IgM showed both bactericidal activity and prozone phenomenon at lower concentrations than IgG The prozone effect of IgM was not found after separation by column chromatography without a prior adsorbtion and elution, but it was readily demonstrable with such a preparation of IgG This was interpreted as a consequence of differences in concentration of the two classes of antibody in hyperimmune rabbit serum, but might have been produced by damaging effects on IgM in the preparation process

Antibactericidal activity of IgM with RO bacteria has never been found The IgM of

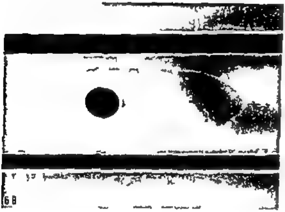

Fig 6 Immunoelectrophoresis of an IgG fraction obtained from ROI 10 (B) and of the whole serum (A) The upper basins are filled with anti total rabbit serum and the lower ones with anti IgG The anode is to the left

ROS–16 was however diluted about 8 times in the chromatography on Sephadex G 200 (a titre reduction from 4096 to 512) which might explain the absence of antibactericidal activity This fraction (Fig 4) contained more protein but had less antibactericidal activity than the IgG-fraction (Fig 2) However determinations were only made on Ig fractions with several specificities which makes conclusions with regard to the quantity of specific antibodies uncertain

In experiments with sheep erythrocytes sensitized with horse amboceptor the haemolysis has been inhibited by certain human sera This protection was caused by natural anti sheep antibodies of the IgG class (19) Consequently, several types of IgG antibodies of different origin and specificity are capable of extinguishing the complement mediated damage to animal and bacterial cells

Chernikh... ostovo et al (1) investigated the immune response to O and Vi antigens of Salmonella typhi in 3 different groups of patients In one group the patients had active typhoid fever in another they were chronic typhoid carriers and patient in the third were vaccinated with killed bacteria They could detect O antibodies only of the IgM class in the vaccinated group both IgM and IgG antibodies in the patients with active disease but only IgG antibodies in the sera of the chronic carriers These observations parallel the antibactericidal effect of IgG antibodies in vitro The appearance of IgG anti

a)

b)

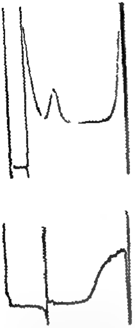

Fig 7 Ultracentrigafugation pattern of two anti bactericidal preparations at 60 000 rpm The direction of sedimentation is from left to right In a) the protein concentration was 3 mg per ml in a sodium phosphate buffer pH 7 3 and μ 0 02 The photograph was taken with Schlieren optics at a bar angle of 50° 50 min after full speed was reached b) The same sample as above after purification on Sephadex G 50 in 0 02 M tris HCl + 0 15 M NaCl buffer pH 7 4 Original conc 0 75 mg protein per ml This pattern obtained after 46 min with ultraviolet absorbtion at 280 nm reveals only one peak with a sedimentation coefficient ($s_{20,w}$) of 6 6 S

bodies in chronic typhoid carriers may, however be just a sign not a cause, of the condition

At present investigations on the mechanism behind the antibactericidal effect are in progress Preliminary results suggests that there is a competition between the normal bactericidal serum factor and specific IgG-antibodies for complement

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BRIEF REPORTS

LONG SURVIVAL TIME OF ISOLATED BALB/c AND DBA/2 MALE MICE

P Ebbesen

It was previously shown that male mice have a much shorter mean survival time than females when grouped sex segregated with 6–8 mice a cage (3) Males living as sole male in cages with eight females, however lived as long as females (3) That male mice living with males are under severe stress was confirmed by *Amkraut and Solomand* (1) who found sarcoma virus induced leg tumours to grow more rapidly in males grouped with males than in males caged as the only male in boxes with females It was furthermore demonstrated (2) that grouping of four males in a cage with six females caused early death of the males indicating that the exposure of males to males and not the sexual isolation is the more important factor

This however still leaves the possibility that the beneficial effect on males of living one male in a cage with several females is not solely due to a shielding from other males Contact with female mice in the box might in itself be beneficial to the male This latter possibility was tested in the present experiment Inbred BALB/c and DBA/2 mice at 4 weeks of age were grouped as given in Table 1 The mice were kept in plastic cages measuring 15 × 30 × 15 *cm* and given chicken pellets and water *ad libitum* All animals were kept in the same room without air condition to ensure a strong odour

Males caged singly and males caged as sole male in cages with castrated males lived as long as males caged as the only male in boxes with females (Table 1)

The beneficial effect of being a sole male in a harem of females thus must be attributed solely to the lack of competing males, without detectable beneficial effect of sexual contacts All animals were exposed to the odour, including pheromones, of stressed and unstressed males (4, 5, 6) but apparently this did not elicit a protracted stress reaction in otherwise shielded males

That the survival time of females is unaffected by grouping is in according with prior experiments (2)

TABLE 1 *Influence of Different Groupings on Survival Time of BALB/c and DBA/2 Mice Kept Simultaneously in One Non Airconditioned Room*

Strain	Grouping in each cage	Number of cages	Sex investigated	Survival time in months Mean (range)
BALB/c	Single male	4	Male	28 (27–30)
	One male with 7 females	4		24 (19–25)
	One male with 7 castrated males	4		27 (25–29)
	6–7 males	2		17 (7–25)
	Single female	4	Female	24 (18–28)
	6–8 females	3		24 (12–28)
DBA/2	Single male	3	Male	27 (25–28)
	6–8 males	3		13 (5–21)

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ESTIMATION OF THYMIC CELL MIGRATION

A New Method

M H Claesson, L Olsson and C Ropke

Thymic lymphoid cells migrate from the thymus and settle in peripheral lymphoid organs (5, 9, 10 12) Only a few authors have considered this problem quantitatively (7, 8, 13) Such experiments involve cannulation of the thymic blood and/or lymphatic vessels thus animals with a cervical thymus (*e g* the guinea pig) or large animals (*e g* the calf) must be used The results have been influenced by the enivitable operative stress—a condition known to induce rapid thymic involution (3) An unknown proportion of emmigrants may belong to the recirculating cathegory, not being newly formed The present work describes a method which allows measurements of cellular migration as well as decay *in situ*, avoiding the inconveniences mentioned above

The experimental animals were young adult female mice from a noninbred NMRI strain The animals were injected intraperitoneally with 1 *μC/g H³ thymidine every 8 hours for up to 10 days* (specific activity 5 0 C per mmol Amersham England) Animals were sacrificed daily eight hours after the last injection of H³ thymidine and 11 13, 15 17 and 20 days after cessation of the injections The thymus was removed and thymus single cell suspensions, nigrosin dye exclusion, cell smears, autoradiographic procedures and analysis performed as described elsewhere (1, 2)

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Neither the cellular compositions of the various thymic smears nor the percentage of nigrosin stained (non viable or decaying) thymic lymphoid cells differed from previous descriptions (2) Fig 1 shows the labelling indices of all lymphoid cells (TL)—viable as well as nonviable—and the labelling indices of the non viable cell fraction (SL) The increase and decrease of labelling appeared to be a logarithmic function of time rather than a liniar one This might indicate that the majority of labelled and non labelled cells emmigrate or decay in the thymus at random (4) After 7 days of H³ thymidine injections about 95 per cent of all the cells (TL) were labelled At this time only

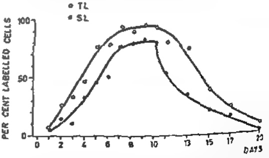

Fig 1 Total lymphoid cell labelling index (TL) and nigrosin stained cell labelling index (SL) as a function of time after multiple injections of H³ thymidine up to 10 days During the first 5 days each point represents the mean value of two mice from day 6 to day 20 each point represents only one mouse

about 85 per cent of the non-viable cells were labelled

The calculations on the daily emmigration of lymphoid cells from the thymus *versus* the decay of cells in the organ are based on the following assumptions *1* The proliferation of thymic blast cells [illegible] constant throughout the period of investigation This is likely as the thymus does not change significantly [illegible] weight during this period 2 The rate of cell decay is constant during the period of investigation This demand is fulfilled as the average cell decay [illegible] 8 4 per cent ± 1 2* 3 The large majority of cells decaying in the thymus is registered by means of the dye exclusion test This is probably the case as the percentage of nigrosin stained cells considerably exceeds that of pyknotic cells *4* Decaying thymic lymphoid cells do not emmigrate Non-viable cells have been shown to be immobile (6, 11) *5* The decaying cells observed at any time are mainly derived from viable cells observed at that time This is likely because grain counts over viable and non-viable cells at a given moment after H³ thymidine injection were found to be on the same level *6* The labelled and non labelled cells do not differ as regards the potencies of emmigration or decay The nearly logarithmic increase in labelling as a function of time fulfils this assumption

The ultimate fate of viable thymic cells is either to emmigrate or to decay *in situ* the difference between the areas below the two curves in Fig 1 indicating the degree of cell emmigration The curves can be divided into three parts an ascending and a descending part and a plateau suggesting different kinetic states of labelled and non labelled cells The emmigration of labelled cells per day is calculated from the equation

$$E_{th} = \left(\frac{1}{n-m}\right) \times \left[\sum_{t=m}^{t=n} P(t) - \sum_{t=m}^{t=n} (TL(t_n) - TL(t_m)) - 8.034 \int_{m}^{n} SL(t)\,dt\right] \quad (1)$$

E_{th} – the emmigration of labelled cells per day
$P(t)$ – thymic cell proliferation at time t after the first H³ thymidine injection
$TL(t)$ – the percentage of all labelled cells at time t
$SL(t)$ – the percentage of labelled non-viable cells at time t

* Standard deviation

By extrapolating the linear ascending part of the curve TL to 100 per cent the turnover time for the thymus is found to be [illegible] days This value gives a daily production rate of new cells of 16 7 per cent Planimetry was performed in order to estimate $\int_{m}^{n} SL(t)dt$ On the ascending part of the curve TL ($m = 0$, $n = 7$) the daily emmigration of labelled cells is 6 0 per cent On the plateau of the curve TL is $TL(t_n) = TL(t_m)$ and the second part of the equation (1) can therefore be neglected At this time interval the daily emmigration is calculated to 9 7 per cent. On the descending part of the curve TL $P(t)$ is eliminated from (1), because at this time no further labelling appeared The daily emmigration of labelled cells at this period was found to be 6 6 per cent On the average the daily emmigration of labelled cells during the experimental period of 20 days is 7 4 per cent ± 2 1 per cent* Thus the average time of cell decay (CS_t, see ref 2) in the thymus is calculated to approximately 22 hours

It must be concluded that the nearly identical values for thymic cell emmigration calculated from the different parts of the curves in Fig 1 strongly suggest the validity of the present method The level of cell emmigration is comparable to values obtained by the more direct methods mentioned in the introductional part of this paper

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LYMPHOCYTE AND HL-A CHIMERISM IN A PAIR OF BLOOD GROUP CHIMERIC TWINS

F Gundolf and *H E Hansen*

The earlier reported investigations of the red cells of a pair of twins in whom blood group chimerism has been demonstrated (2) have now been extended to investigations of the HL-A system Chimerism within the HL A antigens on the lymphocytes of the chimeric twins has been demonstrated Previous attempts by other authors to prove HL A chimerism have been unsuccessful (1) Lymphocyte chimerism concerning XX/XY chimerism has been reported (5), (6)

The investigations were made using the lympho cytotoxic microtechnique (3) Besides the lymphocytes from the twins TS and MR, lymphocytes from the wife (BS) of TS and their son HS were tested and from the husband (ER) of MR and their daughter HR

TS, MR and their family were tested for the following HL A antigens HL AL, HL A2 Ba* (= W28), HL A3, HL A11, HL A9, HL A10 Da25 [illegible] HL A5, TT [illegible] W27), BB [illegible] (= W15), Maki (= W11)

The majority of the test sera used came from multiparous women and the remainder from immunized volunteers

Two to four test sera were used for each antigen and, with the exception of the TT antigen, it was possible to test all the antigens listed above with at least one monospecific serum The HL A antigens demonstrated on the lymphocytes of TS and MR were tested with specially selected test sera which gave only positive reaction (i e 75 100 % of the cells were stained by trypan blue) with lymphocytes carrying the corresponding antigens, whereas a negative result (i e less than 1 % of the cells were stained) was obtained with lymphocytes lacking the corresponding antigens

The anti TT has a special status The antigen TT is closely linked to the HL A12 antigen and anti-TT has only been found in sera containing anti-HL A12 (4) Three sera containing anti HL 12 and anti TT gave positive reactions with TS and MR cells, but two monospecific anti HL A12 sera both gave negative results with the lymphocytes of TS and MR Thus TS and MR must be HL A12 negative and TT positive

Table I shows the strength of the reactions of the test sera which reacted with the lymphocytes of TS and MR Inactivated AB serum from a donor without lymphocytotoxic antibodies was used as negative control Less than 1 % of the cells was stained by trypan blue

The genotypes of TS and MR can be decuded from Table 1 The genotypes were confirmed by HL-A typing of the members of the family The results of the HL A typing of the members of the family are shown in Table 2

Comments

The test sera used in the lymphocytotoxic test (Table 1) were selected in order to give less than 1 % killed cells with lymphocytes lacking the corresponding antigens and more than 75 % killed cells with lymphocytes carrying the corresponding antigens For that reason, TS must have the antigens Ba* HL A9, LND and TT because 75 % or more of his cells are killed by the corresponding antiserum Likewise MR must have the antigens HL-A2 Ba* and TT

Since reactions of the anti LND and the anti HL A9 sera against the lymphocytes of MR gave only 20–35 % killed cells, a cytotoxic reaction with a small population of (HL A9 + LND +) cells must be involved This agrees with the fact that the brother had the haplotype HL-A9, LND The reactions of the anti HL A2 sera against lymphocytes of TS giving 20 35 % killed cells must also be due to a cytotoxic reaction with a small population of HL A2 positive cells and this agrees with the fact that the sister had the HL A2 antigen on the surface of the lymphocytes

The chimerism should also imply that living

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ABLE 1 Sera Giving Positive Reactions with the Chimeric Twins TS and MR in the Lymphocytotoxic Microte

Serum	Specificity	TS cells	MR cells	Control cells Positive	Control cells Negative
B	O	—*)	—	—	—
c	HL-A2 HL-A13	+	+ + +	+ + + , + + + + (+)	— —
9c	HL-A2 HL-A8	+	+ + + (+)	+ + + (+), + + + + + +	— —
3c	HL-A2	+ (+)	+ + + (+)	+ + + , + + + +	—
8c	HL-A2	n d	+ + + (+)	+ + + (+)	—
3500	Ba*	+ + + (+)	+ + +	+ + + , + + + +	(+), + with HL-A – with others
)2 59 7-01-25 02	HL-A9 HL-A1	+ + +	n d	+ + + +	— —
0? 50-7-01-25-01	HL-A9 HL-A12	+ + + (+)	n d		
CLB_4	HL-A9	+ + + (+)	+ (+)	+ + + (+), + + + +	(+) with HL-A1 – with others
\190/70	HL-A8	n d	+ (+)	+ + + +	(+) with HL-A8 and Li — with others
CLB_{10}	LND	+ + +	+	+ + +	—
1192	LND	n d	+	+ + +	—
12714/69	HL A12 TT	+ (+)	+ (+)	+ (+) + (+)	— —
T627	TT HL A12	+ + + (+)	+ + +	+ + + (+), + + + + + + + +	— —
, 30a	HL-A12 TT	+ + (+)	+ + +	+ + + (+) +	—

* Score values — = ≦ 1 % dead cells (DC), (+) = 2–15 % DC, + = 15–30 % DC, + (+) = 30–40 DC, + + = 40–60 % DC, + + (+) = 60–75 % DC, + + + = 75–85 % DC, + + + (+) = 85–98 % I + + + + = 99–100 % DC n d – not done

cells were in excess as compared with the control, when MR is tested against HL-A2 test serum and that living cells were in excess, when TS is tested against HL-A9 and LND test serum In our opinion however the quantitation is hardly sufficiently reliable to justify such conclusion or any inferences to be drawn

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TABLE 2 HL-A Genotypes of the Chimeric Twins TS and MR and Their Families

Person	HL-A genotypes
TS, male twin	Ba*, TT//HL-A9, LND
BS, his wife	HL-A2, SL map1//Da25, HL A13
HS, their son	HL A9, LND//Da25, HL A13
MR, female twin	Ba*, TT//HL A2,x
ER, her husband	HL-A2, HL A12//HL A3, HL A7
HR, their daughter	HL-A2, HL A12//Ba*, TT

x = an unknown antigen in 2 locus

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CONCENTRATION OF HUMAN ANTI HL-A ANTISERA

H E Hansen and *B Rubin*

Sera from multiparae are an important source of cytotoxic HL-A antibodies for tissue typing However, these antibodies are often too weak to be of any use In many cases also the titre of anti HL-A antibodies decreases rapidly after delivery Preliminary results from this laboratory as well as the findings by others (3 6) had shown that the cytotoxic antibodies are mainly of the 7S antibody class An attempt was therefore made to cause weak anti HL A antisera to become useful for tissue typing by isolation, purification and concentration of the 7S antibody fraction of such antisera

It would seem reasonable that the natural ion balance must be maintained in the concentrated antiserum if the serum is to be of use for tissue typing Absorption/elution experiments have been tried with success, but only on very small serum samples (1) The present experiments describe a simple method, using ammonium sulphate precipitation followed by gel filtration as concentration and purification device

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MATERIALS AND METHODS

Sera All antisera were taken from women who had given birth 1-8 months before they were bled The sera of the women were also examined on the 35th week of the last pregnancy as well as 1-2 weeks after delivery All the serum donors had at least three children Sera were inactivated for 30 min at 56° C and stored at —22° C, after adding 1 drop of 0 1 per cent NaN_3 in saline per 10 ml serum

Fractionation and concentration To one volume of human serum was added ½ volume of saturated ammonium sulphate (AS) and the mixture was stirred at room temperature The pH was adjusted to 7 8 Moderate stirring was continued for 2 hours in the cold The precipitate was then isolated by

centrifugation at 2000 g for 30 min and dissolved to the original serum volume by saline The AS precipitation procedure was repeated once, and after centrifugation the precipitate was dissolved in $^1/_4$ to $^1/_5$ volume of saline As judged by immunoelectrophoresis, the precipitate contained γ globulins and traces of β globulins only

The dissolved AS precipitate was filtered through Sephadex G200 as described previously for mouse antisera (4) The following modification were employed 1) Saline was used as eluent in order to be able to detect traces of AS by means of a 2 per cent BaCl solution and 2) no serum was added to the fractionation tubes The fractionation curve revealed two peaks, the first representing 19S γ globulin and β globulin and the second 7S γ globulin (none of the peaks contained AS) The first half of the first peak and the second half of the second peak were collected and concentrated as source of 19S and 7S γ globulin, respectively

Concentration was performed by negative pressure dialysis For pilot experiments, 19S and 7S fractions were concentrated to the original sample volume, and for the actual concentration experiments the 19S and 7S fractions were concentrated to ½ or ¼ volume The total concentration ratio of 19S and 7S antibodies was then 4 to 20 times

This method was applied to human anti HL A antisera (of known titre and specificity) in volumes ranging from 5 0 to 50 0 ml

Test for antibody activity The sera and the fractions were examined by the lympho cytotoxic micro test of *Kissmeyer Nielsen & Kjerbye* (2) The lymphocytes were obtained from volunteers

TABLE 1 *Specificities and Titres of Cytotoxic HL A Antibodies of Serum Samples Taken during 35th Week of Pregnancy and 1 -8 Months after Delivery Compared with Fractionated and Concentrated Samples*

Serum No	Specificities of antibody				Titres of antibody			
	35th week	After delivery			35th week	After delivery		
		Unfractionated	19S	7S		Unfractionated	19S	7S
5d	HL A10	0	0	HL-A10	1	0	0	2
				FJH		0	0	1
77c	HL-A13	0	0	HL-A13	2	0	0	4
4d	HL-A2	HL-A2	0	HL-A2	2	2	0	32
	HL-A13	HL-A13		HL-A13	2	[illegible]	0	8
17c	HL-A11	HL-A11	0	HL A11	2	2	0	8
	HL-A3	HL-A3	0	HL-A3	2	2	0	8
				HL-A10	0	0	0	2
18c	poly*	poly*	HL-A12	poly*	1 128	1-128	4	1 2048

* At a concentration of 1 64 this serum showed only anti HL A12 specificity

TABLE 2 *Concentration Ratio of Sera*

Serum No	Volumes in ml				Concentration ratio**	
	Start	After AS ppt*	After pressure dialysis			
			19S	7S	19S	7S
5d	12	6	2 0	2 2	6	6
17c	12	4	1 5	1 6	9	9
4d	12	4	1 9	2 2	6	[illegible]
77c	6	1 5	1 7	1 7	4	4
18c	8	2	2 0	1 3	4	[illegible]

* AS ppt = ammonium sulphate precipitation
** Approximate values

from the staff of the Blood Bank, and the cell preparation was that used by the same authors (2) After the last washing, the lymphocytes were suspended at a concentration of 2500–3000 cells per μl in a 1 1 mixture of undiluted human AB serum and unabsorbed, undiluted rabbit serum All the AB serum for these experiments was taken from one donor known to have no cytotoxic anti HL-A antibodies It was stored in liquid N_2 The rabbit serum was taken from three to five rabbits and stored in 1 ml samples in liquid N_2 Complement was thawed immediately before use

Background The number of dead cells in preparations with negative control sera was determined by the trypan blue exclusion method to be less than 1 per cent However, corresponding control experiments for testing the serum fractions revealed a background of 1 5 per cent of dead cells For titration, inactivated AB serum was used as dilution medium Before use all sera and fractions were centrifuged in the Spinco microfuge for 4 min at 6000 *g*

RESULTS AND CONCLUSIONS

The sera for these concentration experiments were chosen on the basis of the following criteria

1) The occurrence in the 35th week of pregnancy of well defined cytotoxic HL-A antibodies In the samples used (from 1–8 months after delivery) some or all of the specificities found at the 35th week had disappeared

2) The titres of the cytotoxic antibodies in the sera used for concentration varied from 0 to 1 512

3) The sera examined included a range of variation from mono-specific antisera (tested by absorption) to strong polyspecific antisera containing cross reacting antibodies which could not be made specific by absorption

Twelve antisera were selected, and the results of five representative antisera are shown in Tables 1 and 2 Table 1 shows the titres and the specificities of the antisera before and after concentration, compared with antisera taken from the same donors one week before delivery (35th week sample) Table 2 gives the physico-chemical data of the five antisera.

As can be seen from the tables the described concentration method can be employed to cause weak anti HL-A antisera to become useful for tissue typing Concentration of anti HL A antisera from 4–9 times increased the antibody titres from about 2–16 times In two cases antibodies with new specificity appeared However, the strength of the new specificity was lower than the original specificity, thus still making the concentrated sera useful in appropriate concentration for tissue typing

The concentrated samples were tested by the complement fixation test (5) but were found to be strongly anticomplementary, for some reason as yet unknown Using the leuco agglutination technique (defibrinated blood) the sera as well as the fractions showed a specificity broader than that obtained by the cytotoxic technique

The advantage of this method is that large volumes of weak sera can be concentrated to a small volume with an antibody activity sufficiently strong to be used for tissue typing

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Acta path microbiol scand Section B 80, 157–159, 1972

THE EFFECT OF AERATION ON THE GROWTH OF *ACHOLEPLASMA (MYCOPLASMA) LAIDLAWII A*

Pertti Varkola

In connection with our studies of the nucleotide metabolism of *Acholeplasma* (formerly *Mycoplasma*) *laidlawii A* (*Varkola* 1970) we have looked for optimal cultural conditions for rapid growth and large yields of the actively growing *A. laidlawii*. Complex Edward medium (1947) and its modifications (*Butler* & *Knight* 1960b, *Razin et al* 1966b) have been those most widely used for cultivations of different mycoplasma. Because aeration is also reported to improve the growth of some mycoplasma strains (*Rodwell* & *Rodwell* 1954, *Neuing* & *McLeod* 1956, *Low* & *Eaton* 1965) we have studied the effect of aeration on the growth of *A. laidlawii A* in the modified Edward medium.

The following were the cultural conditions for A. laidlawii A*). The *Butler* & *Knight* (1960b) modification of the original Edward medium (1947) was used with the following substitutions: heart infusion to 1 per cent peptone broth (Evans), 20 vol. of horse serum to 10 vol. of human serum (inactivated by heating for 30 min. at 56°C) and the concentration of penicillin G in the final medium was 200 IU/ml. Furthermore, 7.5 g/l glucose according to *Razin et al.* (1966b) was added to the medium. 24-hour-old *A. laidlawii* culture suspension was used as the inoculum (0.5 per cent) for growth experiments performed at 37°C. The absence of bacterial contamination was controlled on blood agar plates. The growth was expressed in the following manner: 1) as turbidity units measured with a Klett Summerson colorimeter (filter S 54 against uninoculated media incubated statically, 2) as living cells estimated with the colony count method for mycoplasma (*Butler* & *Knight* 1960a), 3) as dry weight, when the cells were centrifuged and washed three times with saline at + 2°C (35 000 × g), lyophilized and dried for weighing in air for 1 hour at 40°C. Three different degrees of aeration were used.

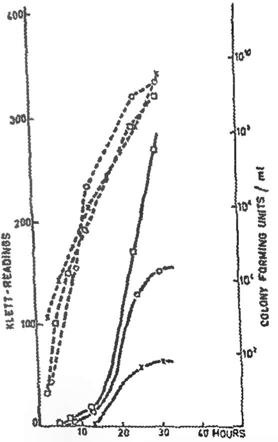

Fig. 1. The effect of aeration on the growth of *A. laidlawii A* measured as colony forming units (dotted lines) and turbidity (solid lines). x "low" aeration (static culture), □ "medium" aeration (shaken culture), o "intensive" aeration (culture in Kluyver flask).

Received 30.xi.71 from the *Central Public Health Laboratory (State Serum Institute), and Department of Virology, University of Helsinki, Helsinki, Finland*

*) *E. A. Freundt*, Institute of General Pathology and Bacteriology, University of Aarhus, Denmark, was kind enough to give us *Acholeplasma (Mycoplasma) laidlawii A*.

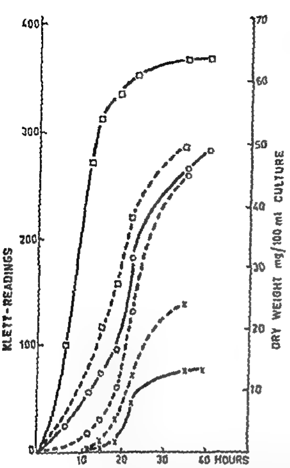

Fig 2 The effect of aeration on the growth of *A laidlawii A* measured as dry weight (dotted lines) and turbidity units (solid lines), x "low" aeration (statical culture), ■ "medium" aeration (shaken culture) and o "intensive" aeration (culture in Kluyver flask)

1 "Low aeration", achieved by incubating cultures statically in 200–2000 ml Erlenmeyer flasks each containing 50 500 ml of the medium
2 "Medium aeration", achieved on a rotatory shaker (150 rev/min) using similar flasks as in the former case
3 "Intensive aeration" was achieved by bubbling sterile air (about 600 ml/min) through 2000 ml Kluyver flasks containing 500 1000 ml of the medium to which 0 5 ml/l of antifoam emulsion (Se, Wacker, W Germany) was added immediately after the inoculum

The growth rate and the final concentration of living cells (CFU) were not significantly altered by different degrees of aeration as shown in Fig 1 On the other hand, the growth expressed in turbidity units or in the dry weight seemed to depend on the degree of aeration (Fig 2) Because of this discrepancy the effect of aeration on the composition of the culture medium and the harvested pellet was studied by means of protein and lipoprotein electrophoresis (*Kaplan & Johnstone* 1966, *Alemens & Schmalbeck* 1969) Furthermore, the harvested pellet (35 000 × g, from a 10 ml sample) was submitted to density gradient centrifugation (4–20 per cent sucrose, 20 000 × g, Spinco SW 50L) at + 4°C for 30 min as a suspension in 0 5 ml saline

A light precipitation was formed even in the uninoculated medium with the "medium" aeration within 24 hours, whereas no precipitation was found with "low" or "intensive" aeration

In the presence of growing A laidlawii both the "medium" and the "intensive" aeration resulted in an almost complete disappearance of the α- and β- lipoprotein fractions from the culture supernatant, whereas the immobile "chylomicron" fraction at the application point of the electrophoresis was greatly increased (Fig 3, D and E) The "low" aeration did not affect the lipoprotein pattern of the medium (Fig 3, C) No change was observed in the ordinary protein electrophoretic pattern of the media during the growth irrespective of the aeration rate

The pellet from "low" aeration culture formed only one heavy band with pure organisms, when submitted to density gradient centrifugation (Fig 3, C) On the other hand, only light fractions were obtained (Fig 3, D) containing high amounts of lipid and cholesterol from the pellet of the "medium" aerated culture Electronmicroscopy revealed vesicular nonmembraneous material among the A laidlawii cells in these fractions In the "intensively" aerated cultures, the pellet formed a heavy fraction of pure organisms (Fig 3 E) in addition to the light fraction

Irrespective of the degree of aeration, the pH of the media decreased during the active growth phase from 8 0 to 7 0 No change in pH was noted in the uninoculated cultures

The variation in aeration did not have any detectable effect on the cell morphology of *A laidlawii A* as revealed in electronmicroscopical studies (*Pirkola*, unpublished results)

These results support the earlier findings of the artificial increase in turbidity of the agitated culture media (*Butler & Knight 1960b*, *Spears & Provost 1967*) One explanation of the phenomenon is that the organism degrades the lipoproteins resulting in an increase of the immobile "chylomicron" fraction This is supported by the observation of *Sethi and Muller* (1970) who found that *A laidlawii A* able to degrade the α- and β- lipoprotein fractions of human serum

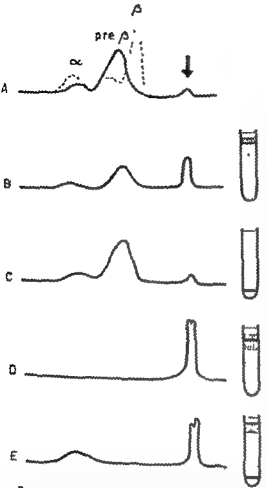

Fig 3 The effect of aeration on the composition of the culture supernatants and on the harvested pellets studied by means of the lipoprotein electrophoresis (curves on the left side) and density gradient centrifugation (tubes on the right side) A uninoculated culture medium (dotted line normal human serum) B uninoculated culture supernatant and the pellet formed after 24 hours of "medium" aeration C D and E Culture supernatant and harvested pellet after 24 hours growth of *A laidlawii A* (C) "low" aeration (D) "medium" aeration (E) "intensive" aeration The application point of lipoprotein electrophoresis is marked with an arrow

On the other hand the phenomenon can be explained simply as follows the agitation of the uninoculated growth medium results in the aggregation of lipoproteins because of their weak solubility, thus changing the lipoprotein pattern slightly

A more pronounced change is observed in the growing cultures because the lipoproteins are also adsorbed on the surface of the organisms This explains the disappearance of the α- and β- lipoprotein fractions from the culture supernatant The formation of the aggregates would also cause the increase in the immobile "chylomicron" fraction of the growth medium, although we have not demonstrated the presence of *A. laidlawii* cells in the "chylomicron" fraction This explanation is, however, supported by the fact that almost all cells in the pellet from the "medium" aerated culture were found in the light fraction formed in the gradient centrifugation In the "intensively" aerated cultures agitation of the medium did not result in aggregation This was probably due to the addition of antifoam, because it kept the lipoproteins in solution better than otherwise seen

Although this aggregation phenomenon did not affect the morphology of the cells it should be taken into account when the growth of *A laidlawii* is to be estimated in different growth media

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SECOND MEETING OF THE SCANDINAVIAN SOCIETY FOR IMMUNOLOGY

Sandefjord, Norway, June 10–12, 1971

Abstracts

K Berg, Institute of Medical Genetics, University of Oslo, Blindern, Oslo 3, Norway POSSIBLE COMPOSITIONAL RELATEDNESS BETWEEN SERUM LIPOPROTEINS AND HISTOCOMPATIBILITY ANTIGEN ON CELL MEMBRANES

We have reported previously that skin grafts survived significantly longer when donors and recipients had the same phenotype within the Lp system of inherited antigens belonging to the lipoproteins of human serum

Metzger has developed a difference index (DI) for assessment of compositional relatedness between proteins based on the over all amino acid composition We have approached the problem of a possible relationship between lipoproteins in serum and histocompatibility antigens on cell membranes by comparing serum lipoproteins with several batches of histocompatibility antigens employing Metzger's method Low values of the DI were found when human histocompatibility antigen was compared with Lp(a) lipoprotein as well as with low density lipoprotein. Lp(a) lipoprotein yielded a very low value even in the comparison with murine transplantation antigen (Science, 172 1136–1138, 1971)

Within the limitations of the method we conclude that the results of the present analysis support the concept that a relationship exists between lipoproteins in serum and histocompatibility antigens on cell membranes

Received 22 x 71

P Bildsoe & M Simonsen, IMMUNOLOGICAL ANALYSIS OF ORGAN GRAFTED RATS WHICH DEFY THE NORMAL RULES FOR REJECTION

Renal transplantation was performed between the AS and AS2 strains of rats which clearly differ at the major H 1 locus Kidneys grafted from AS2 to AS recipients were rejected acutely whereas AS kidneys showed prolonged survival in AS2 recipients in spite of the fact that skin grafts in the same strain combination were rejected acutely The possibility that the long surviving AS2 recipients of AS renal grafts had become immunologically tolerant of the AS strain has been suggested by Salaman, and was supported also by our findings of prolonged survival of AS skin grafts in surviving kidney recipients

As a more stringent test for specific tolerance we analysed the reactivity of the recipient spleen cells by the GVH popliteal lymph node assay in specific as well as third party F1 hybrids A slight decrease in reactivity compared with that of normal AS2 controls was found but the cells reacted clearly differently from those of AS2 rats rendered tolerant of the AS strain in the classical way The conclusion is that specific immunological tolerance is not the reason for the prolonged survival of the renal grafts whereas immunological enhancement so far remains compatible with the findings

I Bjork F A Karlsson & I Berggård, Institute of Medical Chemistry University of Uppsala Sweden, INDEPENDENT CONFORMATIONS OF THE VARIABLE AND CONSTANT HALVES OF A LAMBDA BENCE JONES PROTEIN

Optical rotatory dispersion and circular dichroism studies of a lambda immunoglobulin light chain

and its variable and constant halves will be reported The two fragments, which have been extensively characterized, were isolated after proteolytic digestion of the λ chain A dichroism band at 217 nm, previously found to be characteristic of all immunoglobulins, was given by both the variable and constant halves In other respects, the fragments yielded clearly different spectra reflecting differences in their conformations Comparisons of the theoretical curves, calculated for an equimolar mixture of the fragments with the corresponding curves measured for the λ chain showed that at most minimal conformational changes accompany cleavage of the polypeptide into its halves This suggests that in the intact light chain, the variable and constant parts exist as independently folded regions

K Borum & N Jonsson, INFLUENCE OF NEONATAL THYMECTOMY UPON CELL-MEDIATED TUMOUR SPECIFIC IMMUNITY IN RELATION TO ROUS SARCOMA VIRUS TUMOURIGENESIS IN RATS

Cell mediated tumour-specific immunity was measured in Rous sarcoma virus (SR RSV) inoculated Wistar/Fu rats by an *in vitro* colony inhibition technique using thoracic duct lymphocytes (TDL) and lymph node cells (LNC) from neonatally thymectomized and sham-operated SR RSV injected rats Thymectomy diminished markedly the hourly TDL output, the effect upon the total LNC yield was less pronounced

Cell mediated immunity was demonstrated with both TDL and LNC and with fairly good parallellity It was demonstrated in the same frequency of animals which had not yet developed palpable sarcomas and in animals carrying small tumours, with no difference between the groups of thymectomized rats as compared to sham-operated rats Only in the group of rats carrying large tumours there was a tendency for the neonatally thymectomized rats to disclose such an immunity in a lower percentage as compared with sham-operated rats

The unexpected results that lymphocytes from neonatally thymectomized SR RSV inoculated rats were as efficient in the colony inhibition test as were the same number of lymphocytes from their sham-operated comparable litter mates are tentatively explained by assuming that the continuous presence of the antigen will induce mitotic activity in antigen sensitive thymus derived lymphocytes, thereby expanding the pool of these cells which was originally limited in the thymectomized animals.

To be published in Cellular Immunology

P Brandtzaeg, Immunohistochemical Laboratory, Institute of Pathological Anatomy, Rikshospitalet, Oslo, Norway, EVALUATION OF IMMUNOFLUORESCENCE WITH SUBSTRATE OF SELECTED ANTIGENICITY

Normal rabbit serum was polymerized with glutaraldehyde (Avrameas & Ternynck 1969) to form an inert matrix. Small pieces of this gel were placed in solutions (0 05–2 ▮ mg/ml) of selected human proteins at 4° C for 1–3 weeks, and subsequently fixed in cold 95 per cent alcohol and embedded in paraffin Serial sections, examined by direct immunofluorescence with rabbit antibodies to human proteins, exhibited uniform distribution of reactive antigen Photometric determination of immunofluorescence intensities, revealed a semi logarithmic relationship to the amount of antigen present in a section

Artificial sections may be used as a simple and convenient substrate in the following immunofluorescence tests (1) To determine the effect of various fixatives on the immobilization and antigenicity of soluble antigens, (2) To establish the specificity and optimal staining titre of antibody conjugates, (3) To examine the validity of blocking and adsorption controls, (4) To serve as the substrate for indirect demonstration of antibodies in human or animal sera, (5) To serve as standards for the evaluation of quantitative aspects of immunofluorescence, *e g*, antibody titration of sera and quantitation of selected antigens in tissue sections

H Brunner, Institute of Immunology, University Vienna, Austria, IMMUNOGENICITY AND SPECIFICITY OF HUMAN COLLAGEN AND CHROMIUMSALTSTABILIZED COLLAGEN

In the course of our studies on immunogenicity and specificity of collagen we obtained two further results of interest

a) A comparative investigation of the specificity of human collagen was performed using haemagglutination, haemagglutination inhibition and immunofluorescent techniques In rabbit antisera to acid-soluble human collagen ▮ fraction of species specific antibodies to human collagen and a fraction of general non species specific collagen antibodies could be demonstrated to be in good accordance by both techniques Calf-, rabbit, rat-, and guinea pig collagen were used as heterologous control preparations (1)

b) To elucidate the mechanism of immunogenicity of collagen, acid soluble collagen was treated with chromium III-complex ions for stabilization of the tripel helix structures Rabbits were im-

munized with non treated as well as with chromium stabilized acid soluble collagen preparations (2)

The antisera were investigated for collagen specific antibodies, as well as for the distribution of the different types of collagen antibodies Immunogenicity of chromium treated collagen is less pronounced than that of untreated acid soluble collagen The immune response was the weaker the greater the amount of collagen bound chromium Explications for these findings are discussed

1 *Steffen, C , M Dichtl, B Knapp* and *H Brunner* accepted for publication in Immunology
2 *Brunner, H , M Dichtl and C Steffen* Z Immun Forschg *141* (1971) in press

J E Clausen, TUBERCULIN INDUCED MIGRATION INHIBITION OF HUMAN PERIPHERAL LEUCOCYTES IN AGAROSE MEDIUM

An agarose plate technique demonstrating tuberculin induced migration inhibition of human peripheral leucocytes, is introduced Leucocytes from Mantoux positive persons showed clearly tuberculin induced migration inhibition when, after preincubation for ½ hour with purified protein derivative of tuberculin (PPD), they were placed on agarose plates without PPD The tuberculin induced inhibition was well correlated to the sensitivity of the cell donor as expressed by the delayed intracutaneous reaction to tuberculin.

Leucocytes from Mantoux negative persons showed no tuberculin induced migration inhibition

Non pre incubated leucocytes placed on agarose plates containing PPD showed none or only slight tuberculin induced inhibition

Agarose medium containing horse serum gave larger areas of migration and more pronounced tuberculin induced migration inhibition than medium containing foetal calf serum

Agarose plate cultures were more sensitive than capillary tube cultures as regards demonstration of tuberculin induced migration inhibition

When agarose plate technique is employed only about $^1/_6$ as many cells are used as with capillary tube technique and when leucocytes pre incubated with antigen are cultured in agarose medium without antigen, the method is very saving in antigen

This paper will be published in Acta allergologica scand

A J Cochran & *E Klein* Department of Tumour Biology, Karolinska Institute Stockholm Sweden STUDIES OF THE MOTILITY OF LYMPHOCYTES LYMPHOBLASTS AND LEUKAEMIC LYMPHOID CELLS

Lymphocytes lymphoblasts and the cells of certain malignant lymphoid neoplasms migrate actively from the open end of capillary tubes (1) We have used this system to examine the motility of cells from guinea pig and mouse lymph nodes at various stages during primary immunization There are clear variations in the migratory activity of these cells during immunization The variations correlate well with the proportion of immunoblasts present in smears from the lymph nodes and with the development of cell mediated immunity as assessed by the inhibition of migration of lymph node-derived cells by contact with the immunizing antigen

The technique has also been used to examine the migration of human leukaemic cells Sera directed against human immunoglobulins inhibit the migration of most chronic lymphatic leukaemic cells indicating the presence of whole immunoglobulin molecules on or immunoglobulin moities in the cell surface membranes Other types of leukaemic cells (acute leukaemias and chronic myeloid leukaemias) are not inhibited by anti immunoglobulin sera or are inhibited to a very slight degree A proportion of sera from leukaemic patients inhibit the migration of autochthonous and allogeneic leukaemic cells

1 *Cochran A J* Tumour Cell Migration Europ J Clin Biol Res *16* 44–47 1971

M Crone, C Koch & *M Simonsen* SEPARATION OF LYMPHOCYTES ON COLUMNS COATED WITH ANTI L CHAIN ANTIBODIES

In order to investigate the nature of receptors on the surface of lymphocytes cells from chickens immunized twice with sheep red cells (SRBC) were passed through a plastic bead column (Degalan V 26) coated with rabbit anti chicken L-chain antibody The cells that passed through the column were tested for rosette formation and for their ability to promote a graft versus-host (GVH) reaction The original cell suspension and cells passed through a column coated with normal rabbit globulin were used as controls

During the passage through the anti L-chain column more than 99 per cent of the rosette forming cells (RFC) were retained while the potency of the cells in the GVH assay was unchanged after passage

The results are in accordance with the hypothesis that the receptors on B-cells (f inst rosette forming cells) are of immunoglobulin nature while the receptors on T cells (responsible for GVH) are of a different, unknown type

THE HUMAN SUBGROUPS A_1 AND A_2

The antigenic difference between secreted human blood group antigens A_1 and A was examined in

animal experiments The response of A like and non A-like rabbits injected with A_1 and A_2 substance suggests that these subgroups share a common antigen A, but differ by an actual antigenic determinant present in A_1 substance Thus both quantitative and qualitative differences are responsible for serologic properties of these subgroups

H Diederichsen, ORGAN SPECIFIC ANTIBODIES IN PATIENTS WITH CHRONIC LIVER DISEASE

The occurrence of antibodies against mitochondria in patients with chronic liver disease is well established These antibodies are organ unspecific.

In this investigation an antibody reacting with morphologically characteristic structures in bovine liver has been found in a group of patients with chronic liver disease of unknown aetiology By subsequent treatment with various enzyme staining methods, the fluorescence was shown to be localized to the bile canaliculi Using human liver in stead of bovine liver as antigen, only half of the sera reacted with bile canaliculi. In almost all sera containing bile canaliculi antibody a prozone phenomenon was found as most of the reactions did not turn positive until the sera had been diluted $^1/_4$ or $^1/_8$ Contrarily ANF often found concomitantly with bile canaliculi antibody, gave positive reactions also with undiluted sera Mitochondrial antibodies against parietal cells in the stomach or kidney tubular cells were also found in a few of the sera with bile canaliculi antibody Sera with such mitochondrial antibodies produced fluorescence staining of small granules in the cytoplasm of the hepatocytes Thus, it may be concluded that antibodies against bile canaliculi are not organ unspecific mitochondrial antibodies of the type previously described but specifically directed against liver tissue

S Elsayed & K Aas Pediatric Research Institute and Pediatric Department Rikshospitalet University Hospital, University of Oslo, Oslo, OBSERVATIONS ON EFFECTS OF PROTEIN DENATURATION ON ITS ALLERGENIC ACTIVITY

Anion exchange chromatography and gel filtration of cod white muscle myogens allowed the isolation of an allergenically active fraction designated DS22 (M W 14 500) This fraction was subjected to reduction and denaturation by 8M urea/β-mercaptoethanol The allergenicity and antigenicity of DS22 was maintained after reduction and alkylation by iodo-acetamide The randomly folded material apparently recovered most of its native conformation by dialysis This was indicated by the retainment of the molecular immunopotency, U V spectral profiles and the electrophoretic characteristics The findings suggest that the allergenic activity of DS22 is not determined by the stereochemical configuration of the protein molecules but by the sequence of certain amino acids This was also confirmed through the iso electric focusing of DS22 and the isolation of allergenic and non allergenic constituents using 8M urea as a disaggregating solvent. One major homogeneous acidic protein (pI 4 75) designated Allergen M was obtained This contained the highest yield and allergenicity among the other constituents The activity of Allergen M was but little affected by the high d c potential and the high urea concentration.

We conclude that the allergenic activity of these allergens is sequentially determined

J Allergy 47 283–291, 1971

T Eskeland, Anatomical Institute, University of Oslo, Norway, ISOLATION OF 7S IgM AND KAPPA CHAINS FROM THE SURFACE OF CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS

Chronic lymphocytic leukaemia cells from some patients carry mu and kappa structures on their surface in sufficient amounts to be demonstrated by fluoresceinlabelled antibodies in fluorescence microscopy Previously we have shown that about 50 per cent of the immunoglobulin structures were liberated by homogenization and consisted of 7S IgM and kappa chains We have now tried to liberate the remaining mu and kappa structures from the membranes by treatment with various salt solutions and detergents Some agents like sodium desoxycholate and potassium pyrophosphate were effective Again 7S IgM and kappa chains were found.

S S Frøland, J B Natvig, E Munthe, P Berdal, A Engeset, S O Lie & P Stavem Rikshospitalet and The Norwegian Radium Hospital, Oslo, Norway, IMMUNOGLOBULINS ON THE SURFACE OF HUMAN PERIPHERAL LYMPHOCYTES

By immunofluorescence staining immunoglobulins were demonstrated on the surface of viable peripheral blood lymphocytes In 54 normal individuals, 9 to 22 per cent of lymphocytes stained for F(ab) (mean 10 per cent) The mean for IgG was 5 per cent (range 2 7) for IgM 6 per cent (range 3 12), and for IgA about 1 per cent or less. Among IgG-lymphocytes, the IgG2 subclass was dominating while IgG1 including Gm(f) and Gm(z), and IgG3 were somewhat less frequent, while IgG4 was usually undetectable

Six male patients with congenital agammaglobulinaemia (Bruton) had no Ig positive lympho

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cytes Their lymphocytes responded normally on stimulation with PHA, PWM, ALS, allogeneic lymphocytes and PPD, indicating normal T lymphocyte function Seven patients with Hodgkin s disease had percentages of Ig positive lymphocytes within the normal range, while several of these patients had subnormal *in vitro* responses on stimulation with mitogens A high percentage of lymphocytes staining for Ig, usually IgM, was found in the majority of patients with chronic lymphocytic leukaemia These patients had also markedly subnormal *in vitro* responses of lymphocytes

The Ig positive lymphocytes demonstrated are thought to be equivalent to B lymphocytes in other species

*T Godal, B Myklestad, D Samuel & B Myrvang**
CHARACTERIZATION OF THE CELLULAR IMMUNE DEFECT IN LEPROMATOUS LEPROSY

The blastogenic response of leucocyte cultures from patients with tuberculoid and lepromatous leprosy has been studied The leucocytes from the two groups were studied simultaneously and cultivated in the same pool of normal human serum While the leucocytes from 19 tuberculoid patients responded quite strongly to *Mycobacterium leprae* after 7 days of culture (average lymphocyte transformation 8 7 per cent), there was a complete lack of response in similar cultures from 17 lepromatous patients (ave 0 09 per cent transformed cells) These results were confirmed by studies on cellular incorporation of ^{3}H thymidine in the cultures from 4 tuberculoid and 4 lepromatous patients

This lack of response was quite specific as leucocytes from lepromatous patients responded in the mixed lymphocyte reaction and also to BCG

The blastogenic response of purified lymphocytes to *M leprae* revealed a similar pattern, *i e* the tuberculoid cells responded well, while again there was a lack of response in the lepromatous group

It is concluded that the lepromatous patients lack circulating lymphocytes responding to *M leprae*, indicating that their immunological defect, as observed in the present study, has some features in common with immunological tolerance

* Presented by Dr B Myrvang

S H Golub & J F Henetson, Department of Tumour Biology, Karolinska Institute Stockholm, Sweden, A MICRO COLONY INHIBITION ASSAY FOR DETECTING CELLULAR REACTIONS AGAINST LYMPHOBLASTOID TARGET CELLS

A micro assay colony inhibition (CI) test has been developed for studying cell mediated immunity to lymphoblastoid target cells The conventional CI test requires that target cells adhere to plastic or glass surfaces and cannot be used for stationary suspension culture target cells The modification used in these experiments takes advantage of the ability of the target cells, but not the effector cells, to grow in discrete colonies in semi solid media

Leucocytes from patients with Burkit's lymphoma, nasopharyngeal carcinoma, and controls were tested in this assay against cultured Burkitt cell lines Almost none of the leucocyte samples showed any ability to inhibit colony formation This may be due to insensitivity of the assay with regard to weak tumor associated reactions, or to a real lack of immunologically active cells in the peripheral blood

This CI test can readily detect cells non specifically activated by phytohaemagglutinin (PHA) CI activity of PHA stimulated cells appears to be correlated to the degree of stimulation, as measured by thymidine incorporation Specific CI activity can be obtained from leucocytes stimulated in culture with allogeneic tumour cells Leucocytes have shown a stronger reactivity against the cell used for stimulation than against different target cells

P I Gaarder & J B Natvig, Institute of Immunology and Rheumatology Oslo 1 Norway NON b', AN ANTIGEN OF HUMAN IgG RELATED TO THE Gm SYSTEM

After gel filtration of rheumatoid serum Th at low pH, a hidden, 'new IgM rheumatoid factor specificity was revealed which detected an Fc antigen antithetic to Gm(b) within the IgG3 subclass In addition, this antigen also occurred on all proteins of the IgG1 and IgG2 subclasses, but not on IgG4

After appropriate absorption two baboon antisera B I and B II, raised against an IgG3 Gm(g) and IgG2 Gm(n−) myeloma protein respectively showed specificity against an antigen with a similar subclass distribution These findings showed close analogy to the distribution of the 'non a and 'non g" antigens and was considered to be evidence for the existence of a non b" antigen Further haemagglutination inhibition experiments showed that the antigen detected by antiserum B I was located on the pFc and Fc' fragments whereas the antigen detected by B II and rheumatoid factor Th was not present on pFc' As Gm (b^1) is the only Gm(b) antigen located on Fc outside pFc', the results indicate that the 'non b antigen detected by antiserum B II and rheumatoid factor Th is , non b^1 Several Gm(b) antigens are located on the pFc' fragment but experiments with selected IgG3 myeloma proteins and Gm(b)

anti Rh antibodies showed that antiserum B-I was specific for the "non b[o]" antigen

S Hammarström & P Perlmann IMMUNOLOGICAL STUDIES OF ULCERATIVE COLITIS IMMUNOCHEMISTRY OF THE COMMON ANTIGEN OF *ENTEROBACTERIACEAE* (KUNIN)—RELATION TO LIPOPOLYSACCHARIDE CORE STRUCTURE*

Serum from patients with ulcerative colitis contain auto-antibodies against colon antigen Purified colon antigen is chemically a glycoprotein of blood group substance type The colon determinant is however immunologically distinct from the A B H and Le[a] and Le[b] determinants Anticolon antibodies in ulcerative colitis may be formed as the result of tolerance breakage through cross reactive antigens from the intestinal microflora We have found a bacterial strain *E coli* 014 which cross reacts with colon antigen as assessed by ulcerative colitis sera or by rabbit antisera to *E coli* 014 Lipopolysaccharide (LPS) preparations of this organism contain large amounts of the "common enterobacterial antigen" (CA) Inhibition experiments demonstrated that CA in *E coli* 014 LPS is responsible for the cross reaction with colon antigen Chemical analysis of *E coli* 014 LPS showed that it contained large amounts of LPS core structure not substituted with O specific side chains CA inhibiting activity could be demonstrated in a low molecular weight fragment isolated by gel filtration of an acetic acid hydrolysate of *E coli* 014 LPS Chemical analysis of the fragment indicated that it corresponded to the score region of *E coli* 014 LPS In addition to phosphate and ethanolamine phosphate, it contained the sugars galactose glucose ketodeoxyoctonate and a heptose but lacked N acetylglucosamine The results provide a chemical basis for further studies of the cross reactive colon determinant of ulcerative colitis

* Paper submitted for publication in J Exptl Med 1971

M Harboe K Hannestad & K Sletten Institute for Experimental Medical Research University of Oslo Ullevål Hospital Oslo Norway DICLONAL AND TRICLONAL MACROGLOBULINAEMIA

Ninety macroglobulinaemia sera were tested for occurrence of multiple monoclonal immunoglobulins Thirteen (14 per cent) of the sera contained more than one monoclonal immunoglobulin The homogeneous macroglobulins were isolated from 3 sera, in each case, individually specific antigenic determinants common to the two components were demonstrated in addition to determinants unique for each component NH terminal amino acid sequence studies in two patients showed that the IgM heterogeneity was due to differences in the primary structure of the variable parts of the κ (case Tö) and the μ (case Næ) chains Macroglobulinaemia appears to be a unique source of homogeneous immunoglobulins from related clones

To be published in Scand J Immunol

H F Hazat THE EFFECT OF THE CARRIER PROTEIN ON THE HUMORAL AND CELLULAR IMMUNE RESPONSE TO THE 2,4 DINITROPHENYL (DNP) LIGAND IN BALB/c MICE

It was initially believed that a homologous carrier is a prerequisite for an anamnestic response to the hapten However, we found that the strength of the secondary humoral response depended on the immunogenicity of the carrier rather than the initial exposure to the primary injection We found the most effective protein for eliciting a secondary humoral response to DNP generally was haemocyanin (HCY), regardless of the carrier used in the primary injection

The data presented here are an extension of the previous studies aimed at characterizing the anamnestic response at the cellular level using a modified localized haemolysin in gel technique Balb/c mice were primed with DNP coupled to a number of different carriers and challenged either with the carrier alone or DNP coupled to the homologous or heterologous carriers

In all cases regardless of the protein used for the primary injection, we found that HCY was the most effective "backbone carrier" for anti DNP challenge resulting in a maximum cellular anamnestic response of 7s PFC The cellular response was compared to the humoral antibody levels In addition, the affinity of the antibody was investigated using equilibrium dialysis

S Helgeland & A Grov ANTIBODIES TO STAPHYLOCOCCAL MUCOPEPTIDE IN HUMAN SERUM AND COLOSTRUM

Staphylococcal mucopeptides were agglutinated by normal human sera human sera with increased anti-staphylolysin titres (Astaph sera), and by human colostrum The eluates obtained from mucopeptides sensitized by serum were found to contain IgG and those obtained from the colostrum sensitized mucopeptides contained IgA together with small amounts of IgG Compared to the eluates obtained from mucopeptides sensitized with normal human serum the eluates obtained

using Astaph sera contained greater amounts of IgG Fab, but not Fc fragments of human IgG, were found to bind to mucopeptide, and complexes of mucopeptide and IgG fixed complement

The experiments and results presented will be published in Acta path microbiol scand under the title Immunochemical characterization of staphylococcal and micrococcal mucopeptides

I Heron, Blood Grouping Laboratory, Aalborg Hospital Nord Denmark THE INFLUENCE OF PREGNANCY ON THE IMMUNE RESPONSE TO HETEROTOPIC HEART TRANSPLANTS

The effect of pregnancy on the rejection time and pattern of heterotopic cardiac allografts was studied in rabbits and inbred rats Female rabbits pregnant by donor unrelated males rejected trans plants within normal periods of time In the rat system recipients were transplanted during 1) intrastrain pregnancy 2) interstrain non donor related pregnancy and 3) during interstrain donor specific pregnancy No significant prolongation of allograft survival time was found regardless of the genetic constitution of the foetuses It is concluded that pregnancy induced immunosuppression can not explain the success of the 'foetal allograft' to survive the period of gestation

Paternal strain hearts transplanted to rats in the early postpartum period enjoyed prolonged survival and some were in excellent condition after 160 days Skin allograft survival was not modified and skin was rejected by rats with long term functio ning hearts

The enhancing effect of pregnancy could not be passively transferred by serum drawn in the early postpartum period

Spleen cells from postpartum rats and from rats with long surviving grafts were studied in GVH reactions and normal reactivity against donor antigens was observed

Results obtained by use of parabiosis and transfer of long term surviving transplants to new recipients will be presented

R J Hill & F M Poulsen THE STRUCTURAL AND PHYSICO CHEMICAL PROPERTIES OF AMIDINATED RABBIT IgG

Anti HSA was isolated from immune rabbit serum using bromo-acetyl cellulose HSA and gel filtration on Sephadex G200 Portions of the anti HSA were amidinated with the following bifunctional reagents Dimethyl dodecanoic acid diimidate or Dimethyladipic acid diimidate

Cystein/papain digestion split the amidinated antibodies into a (Fab)₂ and an Fc fragment whereas enzymatic digestion of normal IgG gave Fab and Fc components

Mild tryptic digestion and peptid digestion gave identical gel filtration patterns for both the amidinated and the normal IgG

The three preparations were reduced with 0 1 M β mercapto ethanol followed by alkylation with 0 12 M iodo acetamide Whereas the normal IgG on chromatography on Sephadex G100 was shown to yield L and H chains the admidinated prepa rations were shown to yield a mixture of intact immunoglobulins, H chain dimers and a smaller amount of free L chains

The preliminary conclusion is that the diimido esters have formed covalent bridge(s) between the H chains in the Fd region, and that a small number of bridges may also have been formed between L and H chains

Reaction kinetic experiments were carried out using the Farr technique The ABCs of the amidinated and normal IgG were identical within experimental error The activation energies were also the same for all three preparations However the reaction rate constants for association were decreased and the rate constants for dissociation decreased

T Hjort & P O Janson THYROGLOBULIN IN CORD BLOOD AND IMMUNOLOGICAL TOLERANCE TO THYROGLOBULIN

The occurrence of thyroglobulin antibody is ap parently to some extent influenced by geographical factors and furthermore it might seem as if circulating thyroglobulin is found most often and in the highest concentrations in areas where thy roglobulin antibody occurs in the lowest frequencies—and vice versa Such indications suggest that immunological tolerance to thyro globulin may exist in a varying part of the popula tion in different geographical regions

To test this hypothesis we have examined the occurrence of thyroglobulin in two comparable series of cord blood sera from Gothenburg and Aarhus—supposed to represent areas with high and low frequencies of autosensitization to thyro globulin, respectively

Thyroglobulin was demonstrated both by hae magglutination inhibition with thyroglobulin coated cells and by reversed haemagglutination with antibody coated cels The two tests yeielded nearly identical results

Thyroglobulin could in fact be demonstrated significantly more frequently in cord blood sera from Aarhus than in sera from Gothenburg Thus among 148 Danish and 117 Swedish sera 68 per

The findings are further analysed and discussed in relation to the hypothesis

J. Holmgren, S. E. Holm, L. Å. Hanson & B. Kaijser, Institute of Medical Microbiology, University of Goteborg, Göteborg, Sweden: AN ANTIGENIC RELATIONSHIP BETWEEN HUMAN KIDNEY, STREPTOCOCCI AND *E. COLI* BACTERIA

By means of double diffusion analyses supplemented with absorption experiments an antigenic relationship between human kidney and three out of nine tested *E. Coli* strains of different O groups of possible significance for the pyelonephritic renal scarring process was shown. Antiserum to bacteria of these strains (serotypes O2 K1 H4, O14 K7 H and O22 K13 H1) precipitated with kidney antigen from three adults and one infant and antiserum to human kidney precipitated with veronal buffer extract antigens from the three *E. coli* strains. The veronal buffer extract antigen from the O2 strain contained at least two such antigenic factors: one related to the O2 lipopolysaccharide and another which was present also in the O22 and the O14 strains in an antigenically identical or related form. No antigens related to the tested *E. coli* strains were found in spleen, liver or AB Rh(+) red cells.

In preliminary experiments no relation between the kidney related *E. coli* antigens and the so called common antigen of Kunin or antigen from human colon was found. Antisera to potentially nephritogenic streptococci however gave two precipitation lines with veronal buffer extracts from the three aforementioned *E. coli* strains, one of which identified with a line formed between these antisera and human kidney antigen.

The results will be published in Int. Arch. Allergy

A. Jakobsen: CHARACTERIZATION OF IN VITRO EFFECT AND IMMUNO SUPPRESSIVE ACTIVITY IN DIFFERENT RABBIT ANTI RAT LYMPHOCYTE SERA

Antisera with different *in vitro* and *in vivo* effects can be obtained by varying the immunization schedule. Antisera have been tested for lymphocytotoxic activity *in vitro* and the ability to prolong skin graft survival have been estimated. Antisera produced with a short immunization schedule contained more lymphocytotoxic activity in the IgM fraction than in the IgG fraction while antisera raised with a more prolonged immunization contained most of the *in vitro* activity in the IgG fraction. These antisera had some immunosuppressive activity but much less than antisera raised with a short immunization schedule. Antisera containing *in vitro* activity only in the IgM fraction have been produced in rabbits treated with azothioprine. These antisera showed some immunosuppressive activity.

To be published in Acta path. microbiol. Scand. Sec. B

K. G. Jensen & I. Heron, Blood Grouping Laboratory, Aalborg Hospital Nord, Denmark: CARDIAC ALLOGRAFT REJECTION IN PRESENSITIZED RABBIT RECIPIENTS

In a group of 20 unmodified rabbit recipients receiving a cardiac allograft, 7 rabbits formed lymphocytotoxic antibodies. The histological features of rejection were identical in all cases independent of antibody formation.

Thirteen rabbits were presensitized against the prospective heart donor by means of skin grafts and spleen cell injections and the majority of these recipients were transplanted in the presence of donorreactive cytotoxic antibodies. Accellerated rejection took place in every recipient, but the rapidity of the reaction and the histological picture did not correlate well with the presence and potency of pre-existing antibodies. Histological findings varied considerably including infiltration of mononuclear cells and polymorphonuclear leucocytes, extensive oedema and interstitial haemorrhage and early degeneration of myofibres. Only one case of hyperacute clinical rejection was observed in which the transplant was rejected within 2½ hrs. Heavy deposits of gammaglobulins were demonstrated by the fluorescent antibody method to be localized in the vessel walls of this transplant.

The paper will be published in Acta path. microbiol. Scandinavia

S. Jormalainen: ANTI HAPTEN ANTIBODIES IN NORMAL MAMMALIAN SERA

In all normal adult sera of eight mammalian species studied it was possible to show antibodies to one or more of the haptens NIP, NP, DNP and oxazolone by the hapten-coupled phage inactivation method. The antibodies were almost totally lacking in germ free piglet sera and there were only very small amounts in colostrum free newborn piglet sera. Normal sera did not markedly inactivate uncoupled bacteriophages

There was a great variation between different individuals and different species in the titres of different antibodies and also in the specificities of the antibodies as tested by hapten inhibition

Most of the antibodies were IgM as judged by 2 mercapto ethanol and ultracentrifugal analysis Some antibody activity was also seen in IgG fractions of normal sera separated by DEAE cellulose chromatography

Grant The Finnish Medical Foundation

F A Karlsson, P A Peterson & I Berggård, Institute of Medical Chemistry University of Uppsala Sweden EVIDENCE FOR THE OCCURRENCE OF TWO COMPACT DOMAINS IN IMMUNOGLOBULIN LIGHT CHAINS

Recent work has shown that immunoglobulin light chains can be cleaved by proteolytic enzymes in the region between the variable and constant parts and that the resulting fragments apparently are globular Fragments consisting of variable or constant portions of a lambda immunoglobulin light chain have now been studied in more detail One constant and one variable fragment were obtained from urine three constant and one variable fragment were isolated from enzymic digests of the lambda chains Characterization by means of molecular weight measurements amino acid analyses peptide mapping and amino acid sequence determinations indicated that the fragments corresponded to halves of the light chain and that they derived from splits within a limited region consisting of eight amino acid residues Physical studies of the fragments by gel chromatography analytical ultracentrifugations and viscosity measurements indicated compact close to spherical shapes Accordingly the lambda chain studied should consist of two globular domains connected by a small exposed region susceptible for enzymic attack Similar studies on normal light chains and their halves suggested that this structure most likely is general among human immunoglobulin light chains

A Kostiala & T U Kosunen Department of Serology and Bacteriology University of Helsinki Finland PERITONEAL CELL MIGRATION INHIBITION AND ANTIBODIES IN GUINEA PIGS IMMUNIZED WITH DIPHTHERIA TOXOID (DT)

Guinea pigs were immunized either with soluble DT (group A) or with DT antitoxin precipitate (group B) in both cases 6 μg in Freund s complete adjuvant.

From 3 weeks on all animals had positive 24 hour skin reactions but 4 hour skin react ons were negative in group B Passive haemagglutinins were found in group A from 3 weeks on in group B low titres occasionally from 6 weeks on Haemolysins occurred only in group A from 4 weeks on. Peritoneal cell migration was inhibited in both groups at 3 weeks and more at later t mes

Some animals in group A were boosted intracutaneously with DT in saline three times This increased haemagglutinins haemolysins and macrophage cytophilic antibody on peritoneal cell surface but skin reactions became negative and peritoneal cell migration was not inhibited any more

Thus peritoneal cell migration inhibition correlated with 24 hour skin reactivity and not with antibody concentration Animals that had negative delayed skin reactions did not show migration inhibition, although peritoneal cells had cytophilic antibody on their surface

L Lindholm & L Rydberg DEVELOPMENT OF PLASMA CELLS DURING ACUTE GRAFT VERSUS HOST REACTIONS

Graft versus host (GVH) reactions were induced in newborn homozygous mice by the injection of adult allogeneic lymphoid cells or in two days old F1 hybride mice by the injection of parental lymphoid cells The donor cells were either thymus or spleen cells It was found that large numbers of plasma cells often developed (most prominently in the lymph nodes) after injection of e ther type of cell Studies of the H 2 antigens carried by the cells in the hosts and of the allotype of immunoglobulins in the hosts during the GVH react on strongly suggest that many of the plasma cells were of host origin At least part of the immunoglobulins formed by the plasma cells probably represent antibodies to host antigens since the injected animals developed a positive direct Coomb s reaction Since Coomb s positivity could also be demonstrated with anti host allotype serum it seems probable that host cells synthesise anti host antibodies during GVH reactions

I Lonroth E Falsen L Lindholm & J Westin Institute for Medical Microbiology Dpts of Bacteriology University of Gothenburg and The Medical Clinic II Sahlgrenska Sjukhuset Gothenburg Sweden THREE CASES OF *HEAVY CHAIN DISEASE*

Three patients named NB MS and AH with heavy chains disease were studied NB and MS had a malignant lymphoma AH no known malignity Serum and urine from the patients contained

a M-component, which reacted with anti IgG serum but not with anti Kappa or anti Lambda serum After purification the molecular weights of the M-components were determined using SDS polyacrylamide electrophoresis and found to be 83 000 (NB), 83 000 (MS) and 73 000 (AH) $S_{0}w$ of native MSS protein was 4 7 after correction to zero concentration Immunological subclass was IgG1 for all three proteins The N thermmal seemed to be blocked in the MS and AH proteins

Cells from a lymph node of patient MS were cultivated Electronmicroscopy showed a heterogeneic cellpopulation with blast cells and plasmacell like cells The cell cultures were followed by immunofluorescens Double staining with fluorescent anti Fab and anti Fc shord that most cultures contained cells reactive with anti Fab/Fc and solely anti Fc One culture had 10–30 per cent of anti Fc and no anti Fab reactive cells and this will be used in further biosynthetic and genetic studies

G McDowell, A Grov & P Oeding The University of Bergen School of Medicine The Gade Institute, Department of Microbiology Bergen Norway ANTIGENICITY OF PROTEIN A AND PROTEIN B FROM *STAPHYLOCOCCUS AUREUS*

Protein A from *Staph aureus* is known to react with the Fc region of normal IgG from man and many other species Results are presented to show that the purified antigen elicits synthesis of rabbit IgG reacting with the Fab region Protein B which is present in crude protein A preparations cross reacts with protein A in agar diffusion when antisera are used Absorption of anti protein A and anti protein B with protein A completely exhausted the sera of precipitins for protein A and protein B In contrast anti protein A absorbed with protein B still precipitated protein A but not protein B

Whereas the line produced by protein A and anti protein A (or anti protein B) spurred over that of normal human serum the line obtained with anti protein A absorbed by protein B was confluent with the human serum line

Both Fab and Fc fragments isolated from immune IgG showed reactivity for protein A

The results suggest that protein A and protein B are distinct entities which share antigenic determinants Moreover protein A contains distinctive determinants which are responsible for binding to the Fc region of IgG

The experiments and results presented will be published in Acta path microbiol scand under the title Reaction of staphylococcal protein A with rabbit immunoglobulins

G H McDowell & A J Grov, The University of Bergen School of Medicine The Gade Institute Department of Microbiology Bergen, Norway LOCAL IMMUNITY IN GUINEA PIG MAMMARY GLAND

In pregnant guinea pigs about 1 week before parturition local immunization of the mammary glands with *Staph aureus* strain Cowan I or with mucopeptide from these staphylococci induced a local production of specific antibody which persisted throughout lactation In immuno-electrophoretic patterns of whey developed using antisera to guinea pig serum precipitin arcs corresponding to IgG IgM albumin and a protein of β electrophoretic mobility were seen The latter component was not readily detected in corresponding serum patterns When an antiserum to guinea pig immunoglobulins was used to develop immunoelectrophoretic patterns of whey a strong arc in the β position and a weak IgG arc appeared The protein of β electrophoretic mobility was the only component in whey samples from glands immunized with killed bacteria which reacted with polysaccharide A in immunoelectrophoresis No reaction with polysaccharide A was obtained for whey samples from non-immunized glands

It is suggested that the protein of β-electrophoretic mobility detected in guinea pig whey is analogous to secretory IgA from human subjects and other species and that local immunization with staphylococcal antigens elicits the synthesis of specific IgA

The results presented will appear in a paper to be submitted to Acta path microbiol scand under the title of Local immunization of guinea pig mammary gland with staphylococcal antigens

T E Michaelsen Institute of Immunology and Rheumatology Oslo Sanitetsforening and Rikshospitalet University Hospitals, Oslo 1, Norway ISOLATION AND CHARACTERIZATION OF FRAGMENTS DERIVED FROM PAPAIN PROTEOLYSIS OF HUMAN IgG

Pure IgG from normal sera devoid of aggregates was incubated with papain (enzyme substrate ratio 1 100) at pH 7 0 without cysteine After gel filtration on Sephadex G 150 a considerable amount of protein was eluted between unsplit IgG and Fc plus Fab This protein material was purified using recycling gel filtration—chromatography on Sephadex G 150

By using several methods like double immuno diffusion immunoelectrophoresis, gel filtration in acid milieu polyacrylamide gelelectrophoresis in the presence of sodium dodecylsulphate and immunosorbent chromatography it was shown to consist of a mixture of three fragments one which tenta

tively could be symbolized by $F(ab)_2$ one which shows only Fc determinants and a strange fragment consisting of one Fab linked to one Fc The sum of the last fragments dominates in this material Further incubation of unsplit IgG this time in the presence of cysteine (0 01M), gave similar fragments but in this preparation, the $F(ab)_2$ fragment dominated Essentially the same results could be achieved by directly incubation of human IgG with papain in the presence of cysteine

E Munthe & J B Natvig Institute of Immunology and Rheumatology Rikshospitalet and Oslo Sanitetsforening University Hospitals Oslo Norway COMPLEXES OF IgG RHEUMATOID FACTOR WITH COMPLEMENT IN RHEUMATOID SYNOVIAL TISSUE

Tissue sections from rheumatoid synovial membranes and nodules were pepsin digested to detect IgG rheumatoid factor by immunofluorescent technique In the tissues clusters of IgG plasma cells not able to fix native or aggregated IgG before pepsin digestion fixed these antigens after enzyme treatment indicating intracellular complexes of IgG rheumatoid factor Furthermore these plasma cells fixed the complement factors C1q and C3 IgG plasma cells from IgG1 and IgG3 myeloma bone marrows and from nonrheumatoid inflammatory tissue were similarly tested but did not show the properties of the rheumatoid cells

In undigested sections deposits and inclusions with IgG C1q and C3 sometimes fixed aggregated IgG This fixation increased considerably after pepsin digestion and then also comprised native IgG In a RA latex inhibition system the reactibility of aggregated IgG with C1q disappeared after reduction and alkylation of the IgG The reactibility with rheumatoid factors was retained When fluorochrome labelled reduced and alkylated aggregated IgG was applied on tissue sections the staining of deposits and inclusions was abolished or markedly reduced The staining of plasma cells was unchanged Thus in the present study the binding of aggregated IgG to tissue deposits was due more frequently to C1q than to rheumatoid *factors*

E Moller, O Sjoberg & O Makela Division of Immunobiology Karolinska Institutet Lilla Frescati 104 05 Stockholm 50 and Department of Serology and Bacteriology Helsinki University Helsinki 29 IMMUNOLOGICAL UNRESPONSIVENESS AGAINST THE HAPTEN NNP IN DIFFERENT LYMPHOID CELL POPULATIONS

Mice which received repeated injections of the hapten NNP conjugated to BSA showed a reduced immune response to NNP when challenged with NNP coupled to horse red blood cells and tested in the LHG Jerné assay with NNP coupled to sheep red blood cells as compared with untreated mice The reduction induced with the highly conjugated NNP_7 BSA was more pronounced than the reduction induced with the lightly conjucated $NNP_{1.5}$BSA

Different results were obtained when tests for the presence of specific antigen binding cells were performed in the unresponsive mice The depression of antigen binding cells was found to be less pronounced than the depression of antibody secreting cells

Experiments carried out with the aid of the antigenic marker theta (θ) present only on thymus derived lymphoid cells revealed that a certain proportion of the antigen binding cells reacting to NNP in immune mice were thymus derived lymphocytes However the proportion of thymus derived antigen binding cells present in the unresponsive mice was significantly lower than that in the immune mice These data are consistent with the assumption that the unresponsive state induced to NNP was more pronounced in the thymus derived lymphoid cell population than in the bursa equivalent lymphoid cell population

Part of these data are in press in the Eur J Immunology

J B Natvig M W Turner & T E Michaelsen In

REGIONS OF IgG

A further mapping of the Gm markers and other subclass specific antigens to *subfragments of Fc* was performed by utilizing isolated pFc fragments corresponding to homology region Cγ3 Gm(a) (x) non a and a new γ4 non a antigen and several Gm(b) markers were *located in* the pFc region In contrast Gm(g), (n) (b¹) and non g appeared to be in the *Cγ2 region* Similar localization experiments were also performed for a variety of subclass specific *antigens*

Limited tryptic cleavage of pFc resulting in the loss of approximately seven N terminal residues had no effect on the genetic markers In contrast degradation with papain yielded *Fc fragments* lacking a C terminal tridecapeptide as well as eight or nine N terminal residues *In Fc* the antigens Gm(a) (x) non a and γ4 non a were lost but several Gm(b) markers were present

By using these techniques together with selected papain digestion to isolate IgG1 and *IgG3* subclass proteins some new information was gained about certain rare Gm gene complexes These complexes appeared to have evolved by recent

gene duplications giving rise to two IgG1 cistrons on one chromosome

J E Niederhuber, Division of Immunobiology, Karolinska Institutet, Lilla Frescati 104 05 Stockholm 50, Sweden AN IMPROVED METHOD OF ANTI MBLA PREPARATION

Extensive absorptions with mouse tissues, especially thymocytes, are required in the preparation of a heterologous antiserum which is specific for the bursa-equivalent (B) lymphoid cells of the mouse The surface antigen marker against which the serum is reactive has been termed mouse specific bone marrow derived lymphocyte antigen(s) (MBLA) by its originators Raff Nase and Mitchison

The production of anti MBLA has been greatly improved by using the purified gamma globulin fraction of the immune serum With unfractionated serum 8–10 absorptions with thymocytes in a ratio of 5–10 vol serum to 1 vol. packed cells were necessary before a serum with no detectable activity against T lymphoid cells was obtained In contrast the gamma globulin fraction was specific for B-lymphoid cells after only 3 absorptions This results in a considerable economic saving in the numbers of animals and the time required for absorptions and repeated testings, which is significant when large amounts of antiserum are required for biological studies utilizing purified populations of T and B lymphoid cells

Submitted to Nature for publication

J E Niederhuber & E Moller Division of Immunobiology Karolinska Institutet Lilla Frescati 104 05 Stockholm Sweden THE ORIGIN OF IMMUNOLOGIC MEMORY TO THYMUS DEPENDENT AND INDEPENDENT ANTIGENS

The purification of thymus derived and bone marrow derived lymphocyte populations in spleens of immune mice was accomplished by specific antiserum. A heterologous antiserum anti mouse specific bone marrow derived lymphocyte antigen (MBLA), was used together with the *allo-antiserum* anti-theta (θ) to separate thymus derived (T) and bone marrow derived (B) lymphocytes The presence of antigen sensitive cells and memory cells in these cell populations was studied in an adoptive transfer system. Sheep erythrocytes (SE) a thymus dependends antigen and coli 055 B5 polysaccharide (CPS) a thymus independent antigen were used Immune spleen cells were incubated with normal serum anti θ or anti MBLA in the presence of complement and transferred to irradiated (650R syngeneic recipients Six days after transfer antibody forming cells (PFC) were assayed by local haemolysis in gel

The adoptive transfer of purified populations of T or B lymphoid cells alone or in combination with normal syngeneic thymus, bone marrow or spleen cells demonstrated that immunologic memory to a thymus dependent antigen (SSE) resides in the T-cells Memory cells to a thymus independent antigen, however were found exclusively in the B lymphoid cells Cell cooperation was a necessary prerequisite for a humoral response to sheep erythrocytes but not for the thymus independent antigen

The above is a portion of a communication submitted to J Exp Med

H E Nielsen SENSITIZATION OF LYMPHOCYTES *IN VITRO*

Peripheral lymphocytes from rats of the inbred Fischer strain have been cultured *in vitro* unstimulated and stimulated with (F × BN) F1 hybrid lymphocytes

After four to six days the reactivity of the stimulated and the unstimulated was compared in a graft versus host assay using as hosts (F × BN) F1 hybrids

There was a slightly enhanced reactivity of the stimulated cells after six days of culture

L A Kidson & N R Rose RESTORATION OF [illegible] IN [illegible] OS [illegible]

The Obese Strain (OS) of White Leghorn chickens is characterized by a high frequency of spontaneous auto-immune thyroiditis Bursectomy performed on the day of hatching or *in ovo* reduces the severity and frequency of thyroid infiltration as well as the occurrence of circulating thyroglobulin antibodies This study was undertaken to further elucidate the role of the bursa for a development of thyroid autoimmunity in OS chickens

OS chickens were bursectomized and X irradiated (650 r) on the day of hatching Cell suspensions from individual bursas were *reinjected intra*peritoneally into autologous chickens immediately after X irradiation Bursectomized irradiated chickens and untreated chickens served as controls At the age of 8 weeks the bursectomized irradiated chickens showed a significantly lower degree of thyroid infiltration as compared with the untreated chickens Haemagglutinating or precipitating antibodies against thyroglobulin could not be demonstrated In bursectomized irradiated and bursa restored chickens the thyroiditis was almost as pronounced as in untreated chickens although the incidence of germinal centres in the thyroid was lower Only one out of nine chickens formed demonstrable haemagglutinating antibodies

These findings suggest a dual role of the bursa,

one essential for a development of thyroiditis and one for a development of circulating auto anti bodies

This paper will be published in Immunology

N K Nilsson The Wallenberg Laboratory Uni versity of Uppsala, Uppsala Sweden
SYNTHESIS AND SECRETION OF IgG BY AN ESTABLISHED MYELOMA CELL LINE

Investigations with human myeloma cells were for long hampered by their very short life span *in vitro* Recently, however when improved tissue culture methods were used three myeloma lines were established *in vitro*—one from an IgGI and two from an IgEL myeloma patient The latter two lines secreted complete molecules of IgE indistin guishable from the myeloma protein synthesized *in vivo*

The rate of IgE production *in vitro* and the cell population proliferation have been studied quan titatively under various tissue culture conditions in short and long term experiment The rate of extracellular IgE accumulation depended on the type of medium used, the cell density and the period of time elapsed after explanation The maximum production rate of $8\,1 \times 10^{-17}$g IgE/cell/48 hr was noticed shortly after explantation at cell densities 10^8/30 ml when special media (F 10 RPMI 1640) conditioned by feeder cells were used

Maximal rate of synthesis correlated with rapid cell growth This correlation may be non specific implying only that the cells secrete immunoglobulin at the highest rate when they are in the best phy siological condition It might also indicate that immunoglobulin synthesis and secretion is restricted to a particular part of the cell cycle (S phase)

B Normann O Stendahl C Tagesson & L Edebo Department of Medical Microbiology The Me dical School Linkoping Sweden THE ANTI BACTERICIDAL EFFECT OF RABBIT ANTIBACTERIAL IgG ON THE KILLING OF SALMONELLA TYPHIMURIUM 395 MRO BY NORMAL CATTLE SERUM

sera Earlier experiments have demonstrated speci ficity for the antisera namely that mutants of the same chemotype and phage pattern showed similar antibactericidal reactions These results indicated that antibodies against bacterial surface structures were involved

Rabbit immune sera obtained after immunization for a short time showed high titre in passive hae magglutination but poor antibactericidal activity whereas immunization for long time gave low hae magglutinating titre and high antibactericidal ac tivity The haemagglutinating activity was eluted first at fractionation on Sephadex G 200 indicating that this serological effect mainly belonged to the IgM immunoglobulin class

After fractionating rabbit antiserum on DEAE-cellulose and on Sephadex G 200 and G 25 the antibactericidal fraction was homogenous when examined by immuno electrophoresis and analytical ultracentrifugation The material gave one precipi tation line against antirabbit IgG and S_{20w} was 6 6 S

G Nymand AMMONIUM HYDROXIDE INACTIVATION OF COMPLEMENT IN SERA FOR MICRO COMPLEMENT FIXATION TESTS

The known micro complement fixation tests re cently reported proved suitable for the determina tion of almost all HL A antigens when very potent anti sera were applied in dilution Anti-complemen tary activity however prohibits the use of undiluted sera and thus, weak antibodies are useless in these test systems In this paper a method of complement inactivation is described which prohibits the rise of anti complementary power in undiluted sera and makes it possible to use nearly undiluted sera in micro complement fixation tests

The described method has proved suitable for the detection of weak complement fixing antibodies against platelets and lymphocytes It was possible to demonstrate the antigen HL A 12 on platelets and lymphocytes by means of the micro comple ment fixation technique

The method is compared with the commonly used method of complement inactivation (56° 30 min)

R Perrault ANTI D HETEROGENEITY STUDIES

The importance of the binding constant of various anti D sera and the effect of pH and ionic strength on the reaction between anti D and erythrocytes has been well documented by Hughes Jones and Pollack Their studies were based on the 125I anti globulin technique which allows a mathematical derivation of the index of heterogeneity of an anti D serum However information is lacking to de monstrate whether the net charge of the reactants (red cell surface + antibody molecule) is a de terminative factor in this antigen antibody reaction The lacking information concerns the iso-electric point of the antibody

By now it has been attempted by a direct ap proach to elucidate this question and it was sti mulated by the observation of the differences in reaction of certain anti D sera in 2 Auto-analyser

methods utilizing different principles of antibody detection These observations were further supported by the recovery of anti D activity after ion exchange chromatography Confirmation was obtained by iso-electrofocusing of DEAE sephadex fractions The high sensitivity of the AutoAnalyser methods permits the rapid screening identification and quantitation of anti D activity recovered from chromatographic fractions

The effect of the antibody iso-electric point in various quantitation methods (AA, Enzyme, IAT) and its role in the heterogenity of an antibody is discussed

L Rydberg & *L Lindholm*, Institute of Medical Microbiology, Dpt of Bacteriology, University of Gothenburg, Gothenburg, Sweden

THE POSSIBLE PRESENCE OF ROSETTE FORMING CELLS AGAINST SYNGENEIC THYMUS CELLS IN THE BLOOD FORMING ORGANS OF THE MOUSE

The possibility that mice contain cells carrying receptors for syngeneic antigens was studied by means of the rosette-cell technique Bone marrow (BM) and foetal liver (FL) of mice were found to contain cells capable of forming rosettes with syngeneic thymus cells but not with syngeneic red blood cells (RBC) Also BM and FL-cells eluted from siliconized glass bead columns showed this capability Th— by pre incub mouse gamr preparation of theta ant gen, and by pre incubating the thymus cells with anti theta serum Pre incubation with anti H2 serum did not cause inhibition Theta antigen was coupled to RBC with glutaraldehyde The BM and FL contained cells capable of forming rosettes with such RBC Pre incubation of the RBC with anti theta serum or the BM and FL-cells with anti mouse gamma globulin serum inhibited the formation of rosettes Finally, *in vitro* culturing of BM cells together with glutar aldehyde fixed syngeneic thymus cells caused an increase of the DNA synthesis of the BM-cells These results suggest that the blood forming organs of the mouse contain cells carrying receptors for syngeneic theta antigen

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The mouse mutant nude (lacking a thymus), has been transferred to germfree conditions and maintained germfree for 9 months so far The life span thus seems extended.

Preliminary haematological studies and skin allograftings have been performed Although conventional nude mice have very low leucocyte counts, no difference between germfree nudes and germfree NMRI mice could be demonstrated The most striking finding in the untreated germfree nudes is the normal percentage of lymphocytes contrasting the pronounced lymphopenia in conventional nudes Following allografting germfree nudes develop a relative granulocytosis This is similarly reflected in the bone marrow Immunoglobulin values are low in germfree nudes and NMRI mice with a slight rise in the former group following allografting Results of the allograftings are controversial Some are accepted right away, some with inflammatory reaction, but the majority are rejected This may be due to an intact population of allo aggressive lymphocytes

This paper will be published in part in Acta path microbiol Scand and Zeitschrift fur Versuchstierheilkunde

A Sletten & *K Hannestad*, Institute for Experimental Medical Research, University of Oslo, Ullevål Hospital, Oslo, Norway MULTIPLE M COMPONENTS IN A SINGLE INDIVIDUAL IMMUNOCHEMICAL STUDIES AND N TERMINAL AMINO ACID SEQUENCES OF μ AND k CHAINS

A serum (Næss) contained two monoclonal IgMK immunoglobulins (slow and fast) with different electrophoretic mobility at pH 8 6 and different solubility in dilute buffers Both proteins precipitated specifically with an acid capsular polysaccharide from K. ozaenae type 11, the Fabμ fragment of the IgM molecule The slow component possessed a higher avidity for the polysaccharide than the fast, as shown by competitive precipitation Bo h ... The Fabμ fragments embodied the structures responsible for the antigenic difference between the proteins The sequence of 13 amino acids from NH_2 terminus of the two k chains was identical and revealed that the variable (V) regions of the L chains (V_L) belonged to subgroup V_kI Both H chain species possessed unblocked glutamic acid residues at the NH terminus, indicating that they belonged to subgroup V_HIII However the amino acids at position 6 differed in the two H chains The results show that the two components although very similar in structure, belong to two different clones with different V region genes but identical C genes

J biol Chem (1971) In press

B Solheim & M Harboe, Institute for Experimental Medical Research, University of Oslo, Ullevål Hospital, Oslo, Norway REVERSIBLE DISSOCIATION OF REDUCED AND ALKYLATED IgM SUBUNITS TO HALF SUBUNITS

IgM subunits were produced from three monoclonal macroglobulins by reduction with mercapto ethanol and alkylation The main subunit peak changed position both by gel filtration through Sephadex G 200 and on density gradient ultra centrifugation when the protein concentration was varied With decreasing protein concentration, the elution volume increased and the sedimentation rate decreased, indicating dissociation of the mole cule The data accord with an equilibrium constant of $K = 2\ 1 \times 10^{-6}$ moles monomer/litre for one subunit $\rightleftharpoons$ two half subunits

V Stejskal, H Perlmann & P Perlmann, Dept of Immunology, Wenner Grens Institute, University of Stockholm, Sweden SPECIFIC CYTOTOXITY IN VITRO OF LYMPHOCYTES PRECULTIVATED WITH ANTIBOY TREATED TARGET CELLS

Purified human blood lymphocytes were incubated with chicken erythrocytes (CRBC) in the presence of inactivated rabbit anti CRBC serum, or normal rabbit serum, respectively After 5 days of in cubation the lymphocytes were washed extensively and separated from the CRBC by centrifugation through a ficoll isopaque gradient These lympho cytes were then mixed with fresh ^{51}Cr labelled CRBC and cytotoxicity assessed by isotope release after various times of incubation Lymphocytes pre incubated with antiserum treated CRBC were strongly cytotoxic to CRBC during the second in cubation In contrast, no cytotoxicity was found if lymphocytes were pretreated with normal rabbit serum and CRBC, with antiserum only, or if the CRBC anti CRBC mixture was added at the end of cultivation Lymphocytes precultivated with CRBC anti CRBC were not cytotoxic for duck erythrocytes (DRBC) Conversely, lymphocytes pre cultivated with DRBC anti DRBC were not cytotoxic for CRBC Experiments were performed with ^{125}I labelled anti CRBC antibodies in order to establish whether or not carry over of antibody from the first to the second incubation could be responsible for the cytotoxicity observed Although such carry-over cannot as yet be rigorously ex cluded, our data are compatible with the specific *in vitro* sensitization of lymphocytes by target cell antigens

Th Tallberg, M Lempinen, L Hjelt, M Turunen, W Paile, E J Jokinen & O Alfthan, Helsinki University Medical Center, Helsinki 25 Finland CANCER-IMMUNOTHERAPY USING AUTOLOGOUS TUMOUR TISSUE INSOLUBILIZED WITH ETHYLCHLOR FORMIATE

Ten patients with inoperable gastric or renal adenocarcinoma have been treated for up to six months

Small protein polymer particles (Th Tallberg, Ann Med exp Fenn 1967 45 477–486) of fresh ultrasonicated homogenates were prepared from the patients' own tumours These particles were injected i d and s c together with tuberculin or Candida albicans antigen, every 2, 3 or 4 weeks (0,25–1 ml/inj = approx 500–2000 million particles) During this relatively short follow-up period, the patients have shown apparent clinical improvement Histologically there is a massive lymphocyte infiltration of all tumours with necrosis of malignant cells Metastases have regressed In patients with massive tumour growth there is a transient lymphopenia after booster injections Micro-agglutinates without blasttransformation are formed in 48 h when autologous buffy coat cells are cultured with the tumour polymer particles of the same patient Neither auto antibodies nor circulating antibodies against the tumour or tumour polymer particles can be detected Complement C4 and C3 levels seem to remain normal

So far complications seem to be due to intestinal adherence and scar formation

T Tallberg & E J Jokinen, Department of Serology and Bacteriology, University of Helsinki Finland FURTHER STUDIES OF THE IMMUNOGENICITY OF PROTEIN POLYMER PARTICLES

Protein polymer particles were made from a variety of different antigenic solutions using ethylchlor formiate (Avrameas S & Ternynck T J biol Chem 242, 1651, 1967) As immunogens they induced in hyperimmunizations only IgA and IgG agglutinating antibodies and also specific delayed hypersensitivity

Polymerization did not make proteins immunogenic in autologous animals Immunization of guinea pigs with polymerized autologous serum with or without incomplete or complete Freunds adjuvant did not produce auto-immune reactions

Heterologous polymer particles used as immunogens from the day of birth did not produce tolerance against the immunogen Antibody active protein polymer particles immunologically and specifically coated at equivalence with an antigen induced only agglutinating antibodies of high titre The specific antigen used with these immunolog

cally coated particles was human placental lactogen, and the amount of ant gen was 5–10 ug/an mal. (Tallberg et al in Prot des of B olog cal Flu ds XVIII Ed Peeters Pergamon Press Oxford.)

Convalescent or antiv ral act e part cles immunolog ally coated with antigen c viral components w ll be tested n vaccination experiments

E Tho sby A Engeseth J Folk & S O Lie
HL-A ANTIGENS AND SUSCEPTIBILITY TO DISEASES*

An mal stud es ha e shown that both the spec f c immune response to some synthet c antigens and the su ceptibil ty to some leukaen ogen c viruses are c osely related to alleles of the major his o compa b l ty locus Th s has caused a search for poss ble correlations between alleles of the human HL A sys em and suscept b l ty to var ous mal g nant and non mal gnant d sorde s

We have stud ed 28 ch ldren w th acute lympho blas c leukaemia (ALL) 117 pat ents w th Hodg k n s d sease (HD) and 34 ch ld en with serious asth ma Our results ind cate an ncreased frequency of the haplotypes HL A2 12 and HL A1 8 in ALL the an gen HL A5 n HD and the haplotype HL A1 8 among as mat c ch ldren The haplotype HL-A1 8 also seems to ha e an increased f equency among patients that ha e completely rejec ed a transplanted k dney A special immune respons veness m ght thus be associated w th this part cular hap otype

* The paper w ll be published n "Tissue Antigens" no 3 1971

H To le haug & K Hanne od Institute for Experimental Med cal Research Un vers ty of Oslo Ullevål Hosp tal Oslo Norway STUDIES OF THE COMBINING SITES OF A MONOCLONAL MACROGLOBULIN WITH MULTIPLE SPECIFICITIES

A monoclonal IgM λ mmunoglobul n E H reacted w h n rophen l groups and with certa n acid c pol mers such as hepar n dex ran sulphate dena ured DNA and polystyrene sulphon c acid but d d not eact with pol glutamic ac d hyaluronate o chondro t n sulpha e In equ l br um d alys s the assoc a on cons ant of both ntact IgM and ts Fabµ fragmen for DNP eps lonam nocap roic ac d DNP EACA) was 2700 l/mol The heterogene ty ndex was 0 93 nd cat g that the s tes were nea ly homogeno s w th respect to b nd ng aff n ty DNP EACA nh b ted the prec p n reaction between intact IgM and all the mac e a id c polymers The immunoglobul n d d no bind urid ne and the assoc at on constants of the haptens 4 n tro and 3 5-d n trobenzo c acid relat ve to DNP EACA (K$_r$) were 0 05 and 0 1 respectively

The results ind cate that the same or overlapping sites on the Fabµ fragment react w th all the various structures It s suggested that the IgMλ E H site contains both cation c and hydrophob c groups and that with certa n l gands e ther group may predom nate in the b nd ng

B Vandvik Dept of Neurology and Institute of Immunology and Rheumatology Rikshosp talet Oslo Norway STUDIES OF IgG IN BRAIN IN SUBACUTE SCLEROSING PANENCEPHALITIS

Immunoh stoch em cal stud es of brain t ssue from a ca e of subacute sclerosing panencephal tis (SSPE) ind cated synthes s of IgG in mononuclear cells as well as b nd ng of IgG to neurons and glia cells

Immunochemical invest gat ons of brain extracts revealed greatly increased IgG content in the SSPE brain (370–450 mg per cent) when compared to normal bra ns (5 13 mg per cent) Furthermore SSPE bra n IgG was composed of several electro phoretically homogenous fractions ident cal to frac t ons in the pat ent s cerebrosp nal fluid (CSF) These differed in the r kappa and la nbda light cha n specif cit es suggest ng monoclonal popula tions of IgG

Brain IgG showed many fold h gher measles antibody t tres than serum IgG The serolog cal data further s ggests that while all monoclonal pop ulat ons were measles ant bod es they may be d rected agai st different ant gen c components of measles v rus

Recent e dence l nks SSPE to a slow brain infection with a measles l ke v rus It is suggested that this induces a state of hyper mmun zation result ng in local synthes s of homogenous ant body and that s milar princ ples of homogenous ant body response may be operat ve n oth er d sease characterized by homogenous CSF IgG fract ons such as mult ple scleros s

G A Vyas Dept Clin Path & Lab Med Uni vers ty of Cal forn a San Franc sco Cal f 941** U S A SEROLOGIC SPECIFICITY AND HETEROGENEITY OF THE HEPATITIS ASSOCIATED ANTIGEN

The detect on of hepat t s a soc ated antigen (HAA) and ts ant body s best achieved by an ind rect haemagglut nation assay using $CrCl_3$ coupled HAA which s de o d of human plasma prote s ob a ned by comb ned sopycn c band ng and ra e sed mentat on ul racentrifugation on $CsCl_2$ grad ents The resul s of a recent study sponso ed by the Nat onal Research Counc l of the

U S A indicated the haemagglutination assay to be be most sensitive and specific for detection of HAA Its practical application is now enhanced by use of a semi purified HAA containing 10 per cent human plasma proteins obtained from second ultracentrifugation run This HAA material can be heated at 60° C for 10 hours without impairing its serologic activity The immunochemical specificity is compensated by incorporation of group AB plasma of Ag(x⁺y⁺) type in the medium for cell suspension The selection of appropriate reagents is crucial to the success of the haemagglutination inhibition assay because 8 per cent of the HAA positive sera are missed by use of certain HAA preparations which have enabled us to define at least two antigenic determinants HAA of one type has been isolated in sufficient amounts and studied for its amino acid analysis, the chemical basis of this serologic variation is currently under study with a hope to define the primary structure of the antigenic determinant

D Hager & J B Natvig Institute of Immunology and Rheumatology, Oslo Norway ENZYME SENSITIVITY AND SEROLOGICAL ACTIVITY OF Clq

Preparations of the human complement factor Clq obtained by precipitation at low ionic strength were subjected to enzyme digestion with pepsin and papain Varying the pH of the incubation mixture and time of exposure to these enzymes has a marked effect on the type of products obtained After less than two hours incubation at 37° C, pepsin treatment of the Clq preparations at pH 4 0 0 1 M sodium acetate buffer almost completely destroyed the ability of Clq to aggregate latex particles coated with IgG, precipitate with DNA or precipitate with rabbit antiserum specific for Clq Milder treatment with pepsin at pH 4 5 0 3 M sodium acetate buffer caused the formation of a split product which lacked some of the antigenic determinants present in the nontreated preparation The ability to aggregate latex particles coated with IgG was also destroyed Papain digestion at pH 5 0 also gave split products and partially destroyed the ability to aggregate latex particles coated with IgG

J A Aarli & O Closs BINDING OF NORMAL Fc FRAGMENTS TO SKELETAL MUSCLE

Fc fragments were prepared by separation on CM cellulose and Sephadex columns of papain digests of γG globulin from normal human sera The purity of the products was controlled by immunoelectrophoresis The antiglobulin consumption test was performed using lyophilized skeletal muscle tissue treated with the Fc fragment preparations Rabbit anti human Fc and rabbit anti human γG globulin served as antisera The antiglobulin consumption observed increased with increasing amounts of Fc fragments used for incubation with the tissue

Indirect immunofluorescence studies were performed using frozen sections of skeletal muscle and fluorescein labelled anti γG globulin With normal Fc fragments, a cross-striational pattern was obtained The striational fluorescence was indistinguishable from that obtained with F(ab)—fragments prepared from sera of patients with myasthenia gravis Thus the demonstration of cross striational fluorescence does not necessarily indicate binding of muscle antibodies to cross striation of skeletal muscle

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A NEW BACTERIOPHAGE TYPING SET FOR *PSEUDOMONAS AERUGINOSA*

1 *Selection Procedure*

TOM BERGAN

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, University of Oslo, Oslo, Norway (Head Professor dr med S D Henriksen)

The selection procedure for a new bacteriophage typing set for *Pseudomonas aeruginosa* is described The lytic spectra of 113 internationally available phages derived mostly from previous typing sets had been grouped by numerical allocating techniques applying the unweighted pair group average cluster analysis to the Jaccard Sneath similarity matrix and the matrix for the transformed Yule correlation coefficient This had rendered dendrograms demonstrating the similarity existing between the lytic spectra of the various phages From each of the clusters arising above a phenon line drawn at a level with approximately 25 clusters one representative phage was selected Accordingly a series of alternative sets were constructed and analysed for their best conformity to the criteria (a) lysis of the largest possible percentage of bacterial strains, (b) good differentiation, and (c) convenient number of reactions per plate On this basis a *new primary phage typing set* for *P aeruginosa* of 19 phages and an *auxiliary phage typing set* of 5 phages were selected

Early works on the bacteriophage typing of *Pseudomonas aeruginosa* suggested marked differences in the lytic spectra of individual phages (16 47) *Potel* (42) reported his experiences in typing *P aeruginosa* with a set of seven phages Since then 12 different sets have been documented in the literature (20 21 22 23 29 30 31, 32, 40 41, 46 50) and additional modifications are known to exist Compared to the results with the international phage typing set for *Staphylococcus aureus* it appeared that the pseudomonas set which has reached the widest distribution (30) and which has been employed in this laboratory, had impractically long pattern codes and left too many bacterial strains untypable The other available alternatives entailed basically the same problems An entirely new and hopefully better typing set could presumably be constructed by suitable selection among the phages of previous sets Conceivably, each of these consisted of phages which had in turn been selected from larger collections on the basis of their superior suitability as typing phages

The purpose of the present communication is to describe the process involved in selecting a new pseudomonas phage typing set based on numerical allocating procedures of the lytic spectra of phages from previous typing sets (6, 7) and to describe a few basic characteristics of this new set

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MATERIAL AND METHODS

Bacteriophages and Bacterial Strains

The 113 bacteriophage strains and 486 strains of *P. aeruginosa* employed have been used in a previous study (6) In this connection, each isolate of *P aeruginosa* was considered as one strain with the understanding that there might, in fact, be duplications The same applied to each of the 113 phage strains of the final set, in spite of the fact that their past history showed that some might have a common origin (Table 1) The similarity of such phages, however, was mostly minor as evidenced by the cluster analysis (6,7)

Identification of P aeruginosa

The criteria for species diagnosis of *P aeruginosa* followed *Jessen* (26) Primary cultures from this hospital were selected for typical appearance and odour on blood agar (Tryptose Blood Agar Base (Oxoid) with 5 per cent oxalated bovine blood or human blood added) All strains were

(a) Gram negative,and
(b) Oxidase positive (Kovacs)

For strains with

(c) Pyocyanine or pyorubrin production (King's medium)

with or without

(d) Pyofluorescein formation (King's medium), the diagnosis of *P aeruginosa* was considered final The remainder were accepted if the following reactions were as indicated in the parentheses

(e) Growth at + 42°C (+)
(f) Reduction of nitrate (+),
(g) Liquefaction of gelatin (in broth with 15 per cent gelatin) (+),
(h) Adonitol (—),
(i) Dulcitol (—),
(j) Inositol (—)
(k) Mannitol (+),
(l) Saccharose (—),
(m) Sorbitol (—)

Cultivation for tests numbers (c) – (m) was done at 37°C for 4 days and further processed according to (26) Discordance in one or two of the reactions (e) – (m) could be compensated by a lack of growth at + 5°C and/or no egg yolk reaction

Culture Medium

For purification, propagation, typing, or quantitation, the following medium was employed Tryptone Difco, 10 g, Yeast Extract Difco, 5 g, NaCl, 5 g, CaCl, 0 4 g, $MgSO_4$ $7H_2O$, 0 2 g, glucose 4 g, 250 ml of a solution containing 24 3 g tris hydroxymethylaminomethane per liter (0 2 M), 225 ml of a 0 2 M HCl dilution (16 6 ml concentrated acid per liter), and distilled water ad 1000 ml The mixture was adjusted to pH 7 1 before autoclaving for 15 minutes at 121°C For the *agar layer method* (*vide infra*) 4 ml aliquots of medium with 0 6 per cent Agar (Difco) were poured on top of 9 cm typing plates containing approximately 20 ml medium with 1 4 per cent Agar (Difco)

Purification

A minimum of three successive clonal isolations were made for each phage strain before stock propagation For purification, the *agar layer* method was used as described by *Adams* (1)

Propagation

Propagation was done in broth with 250 ml contained in flat bottom Erlenmeyer flasks incubated in a gyratory shaker at 37°C for 5 hours Quantitation was done by optical density (O D) measured on a Beckman Model C Colorimeter with green filter of transmission in the range of 510 – 580 nm The propagation flasks initially contained approximately 10^7 cells per ml of an overnight broth culture of *P aeruginosa* Phage was added to a multiplicity of infection of 1 plaque forming unit (PFU) per 100 viable bacterial cells This set up had been found to be optimal in preceding tests with the phage host systems 7B, 44, 73, M4 and Col 21 examining phage yield with 10^{3}, 10^{2} 10^{1}, and 1 PFU s per bacterium, after different intervals

Typing Procedure

The typing procedure and scoring of the lytic reactions were as in staphylococcal phage typing (11) The plates were flooded with an even sus- [illegible] approximat [illegible] (Fi [illegible] from overnight blood agar cultures Taking cu [illegible] d rectly from plates was inspired by studies on stability of staphylococcal phage types when the bacteria were grown in broth (45)

Numerical Procedures

The Jaccard Sneath similarity index (S_{JS}), the transformed Yule correlation coefficient (S_{φ}), and clustering analysis have been presented previously (6,7) The terms [S_{JS}] UWPGA, [S_{φ}] UWPGA and other concepts of numerical allocating techniques have been explained before (6,7)

RESULTS

Examination for Purity of Bacteriophage Strains

Most of the phage samples received rendered plaques of homogeneous morphology Although the differences in some instances

TABLE 1 *Presentation of the* Pseudomonas aeruginosa *Bacteriophages Studied in the Selection of a New Set* §)

Number code *)	Strain designation †)	Received from	Inclusion in typing sets	Source of original isolation **)	Lytic activity ‡)	Comments
1 13	2 7A 8 16 21A 21B 24 31A 31B 44 68 73 109	RBL	G HD RBL	s	37 39 39 10 40 44 12 19 17 27 36 5 23	Received from HD by RBL
14	119x	RBL	RBL	s	26	Isolated from Melbourne sewage by BWH
15–16	352 1214	RBL	G RBL	1	16 35	Isolated by G
17 19	F7 F8 F10	RBL	RBL		7 25 04	Isolated by PF?
20–21	M4 M6	RBL	RBL		11 4	
22 24	Col 11 Col 18 Col 21	EA	CIRL	1	?6 30 5	Isolated in CIRL where used in conjunction with phages 2 5 7–8 10–24 Col 11 = PK 12 and isolated by PK Col 21 = VLS x C21
25	Pc	CGM		1	5	Isolated by L Dickinson cfr reference (15) Propagating strain C10
26–39	1 2 3A 3B 4 5 6 7 8 9 10 11 12 13	EM	EM	1	24 29 ?7 16 31 34 26 ?8 34 26 26 10 3? 23	Phages elsewhere indicated by prefix Me Propagating strains 72 79 79 79 237 406 1160 184 280 95 50? DD 390 411
40–43	3 11 95 113	JBG	RBL HD	s	36 5 1 8	Isolated by HD
44–59	[illegible] F116 D3C L (A) D3C'L (B) E79 G101	BWH		1	29 5 1 1 29 2	Used for typing but not as a formal typing set
50–5?	103g 176p 188/1	JDP	JDP	1	1 2 27	Isolated and used by JDP together with phages 2 5 7–8 10 24
53 5	Px2 Px3 Px5 Px7	RHO		s	1 17 25 21	Propagating strains Pa32 Pa38 Pa31 Pa31 135
57	2A 2B 3 4 5 6 8 9 10 12A 12B	EA	PK CIRL VLS	1 2 3 4 9 10 s 5 6 7 8 11 12	25 24 1 ? 32 20 34 23 ? 32 32	Phages are indicated elsewhere by prefix K Propagating strains 45 45 14 45 30 109 167 109 20 30 30
? 73	C1r C1r C3 C4 C7 C9	VLS	PK	1	29 17 03 7 32 25	Isolated by PK Sent to VLS by EA C1c and C1t are substrains of C1
? 79	C13 C15 C16 C19 C21 C?2	VLS	CIRL VLS	1	6 19 6 5 23 6	Isolated in CIRL Sent to VLS by EA C21 = Col 21 Strains C16 and C22 propagated on *P aeruginosa* strain 16 22
80–8?	H95 H116 H?49	VLS	HD VLS	1 H249 s H95 H116	13 29 21	H249 sent to HD by G and from HD to VLS
81 89	P2 P6, P? P8 P9 P10 P12	VLS	PF	1	20 6 16 3	Isolated by PF

TABLE 1, *continued*

Number code *)	Strain designation †)	Received from	Inclusion in typing sets	Source of original isolation °)	Lytic activity ‖)	Comment
90–102	I, III, IV, V, VI, VII, IX, X, XI, XII, XIIIA, XIIIB, XVI	HCZ	HCZ	l	17 [illegible]	[illegible] Isolated by HCZ
103–113	1, 2, 3, 6, 9, 16, 18, 19, 20, 21, 22	HCZ	CIRL, HCZ	l	21, 22, 3, 28, 33, 12, 32, 9, 5, 4, 6	Propagation phage 2 on pseudomonas 2/4, ø6 on ps 6/10, ø16 on ps 16/22 Isolated in CIRL Sent to HCZ by EA Phages indicated elsewhere by prefix Z

§) Abbreviations

1) Received from

EA = Dr Elizabeth Asheshov, Cross Infection Reference Laboratory, Colindale London, England
JBG = Dr J B Grogan, University Medical Center, Jackson, Miss 39216, USA
BWH = Dr B W Holloway, Department of Genetics, Monash University, Clayton Victoria, Australia
EK = Dr E H Kaklamanis, 8 Karneadou Street, Athens 139, Greece
RBL = Dr R B Lindberg, US Army Surgical Research Unit, Fort Sam Houston Texas 78234, USA
RHO = Dr R H Olsen, Department of Microbiology, Medical School The University of Michigan Ann Arbor, Michigan 48104, USA
CGM = Mr C G Mason, Biological Sciences, Research Department, Boots Pure Drug Company Ltd, Nottingham 56255 England
EM = Dr Eugenia Meitert, Institutul de Microbiologie, Parazitologie si Epidemiologie Dr I Cantacuzino', Spl Independentei 103, Bucharest, Rumania
JDP = Dr J D Piguet, Section de bactériologie, Institut d hygiene, 22 quai de l Ecole de Médecine Geneva, Switzerland
VLS = Dr Vera L Sutter, Veterans Administration Center, Wilshire Boulevard Los Angeles, California 90073, USA
HCZ – Dr H C Zanen, Gemeente Ziekenhuis, Wagnerlaan 75, Arnhem the Netherlands

2) Typing sets

CIRL – Cross Infection Reference Laboratory During 1961–1962 36 phages were selected at CIRL. Thirteen of these phages and the PK phages except phage 6 were used at CIRL from October 1962 The set of 13 phages were sent to VLS under the designations C12 C24
EM = Meitert, reference (31)
G = Graber *et al* (21)
HCZ – Zanen (50)
HD = Hoff & Drake (23)
JDP = Refers to a set of phages 5, 7 8, 10–24, and 50–52
PF = Postic & Finland (41)
PK = Pavlatou & Kaklamani (40)
RBL – Lindberg *et al* (29 30)
VLS = Sutter *et al* (46)

*) [illegible] previous papers (Bergan 1971ab)
†) [illegible] (A)–D3C L⁺(B), K2A 2B K12A 12B, were from same

°) s = sewage, l = lysogenic strain
‖) Indicates the percentage of strains lysed among 486 strains of *P aeruginosa*

were but slight, the following phage pairs were obtained from the same aliquots 2–3, 5–6, 8–9, 28–29, 46–47, 57–58, 66–67, 100–101 (code numbers as explained in *Table 1*) *The Sjs and Sp* indices expressing the host range similarity for these pairs and the per

TABLE 2 *Comparison between Bacteriophages Isolated from Same Phage Samples*

Phage pair	Phage code numbers	S_{JS}	S_p	Percentage of lysis	Evaluation of phage pair similarity
a	2 3	98	99	37 39	Identical
b	5 6	88	95	40 44	Identical
c	8 9	56	83	19 17	Dissimilar
d	28 29	25	63	27 16	Dissimilar
e	46 47	33	75	1 1	Possibly identical*)
f	57 58	87	95	25 24	Identical
g	66 67	36	66	32 32	Dissimilar
h	100 101	88	97	5 5	Identical

*) The relatively few reactions exhibited by these phages make a conclusion uncertain

cent lysis conferred upon 486 pseudomonas strains suggest that four pairs consist of in distinguishable, three of dissimilar, and one possibly of identical phages (Table 2)

Process of Set Selection

As candidates to the phage typing set, phages which lysed approximately 10–15 per cent of the bacterial strains were preferred Phages rendering a clear cut, easily detectable reaction were chosen to reduce day to-day and observer to-observer variation

Selection of the final set was guided by the results of the numerical analysis of the phage lytic spectra as achieved by the unweighted pair group average cluster analysis (UWPGA) based on S_{JS} and S_p In each of the corresponding phenograms (6, 7) a phenon line was drawn across cluster stems, such that approximately 25 phenons emerged The resulting subdivisions and some of the cluster properties are indicated in Tables 3 and 4 Selecting one representative phage from each cluster would presumably, if clusters were homogeneous, result in a typing set with diversification in lytic spectra

Due to the size of some of the clusters (Tables 3 and 4), several alternative sets could be selected In the first attempt, 22 different sets were constructed A set based on [S_p] UWPGA (7) was found to satisfy these criteria best and was further tested in a series of permutations Finding the set which best fulfilled the characteristics outlined below followed a trial and error procedure with various permutations of the sets which showed the "best" results during the first attempts In all, 73 different alternative sets were analysed for their suitability as tested against the phage reactions on the 486 strains which previously had formed the basis of the numerical analysis of the phage lytic spectra

As aids in the process, charts were made up to identify the bacteria not lysed by the experimental sets and the other phages attacking these, and the similarity matrices for the sets In the controls done by punch cards attention was centered upon (a) percentage of strains typed (b) number of different phage patterns, and (c) mean number of reactions per plate The results obtained by testing alternative sets are demonstrated in Table 5 on the hand of some examples The set selected as the most suitable one was found to consist of

TABLE 1, *continued*

Number code *)	Strain designation †)	Received from	Inclusion in typing sets	Source of original isolation *)	Lytic activity ‖)	Comment
90–102	I, III, IV, V, VI, VII, IX, X, XI, XII, XIIIA, XIIIB, XVI	HCZ	HCZ	l	17, 2, 2, 32, 7, 7, 6, 1, 11, 12, 5, 5, 14	Propagating strains 4325, 7502, 4325, 4325, 5803, 4325, 6471, 6471, 6471, 6471, 7365, 143 Isolated by HCZ
103–113	1, 2, 3, 6, 9, 16, 18, 19, 20, 21, 22	HCZ	CIRL, HCZ	l	21, 22, 3, 28, 33, 12, 32, 9, 5, 4, 6	Propagation phage 2 on pseudomonas 2/4, ø6 on ps 6/10, ø16 on ps 16/22 Isolated in CIRL. Sent to HCZ by EA Phages indicated elsewhere by prefix Z

§) Abbreviations

1) Received from

EA = Dr Elizabeth Asheshov, Cross Infection Reference Laboratory, Colindale London, England
JBG = Dr J B Grogan, University Medical Center, Jackson, Miss 39216, USA
BWH = Dr B W Holloway, Department of Genetics, Monash University, Clayton Victoria Australia
EK = Dr E H Kaklamanis, 8 Karneadou Street, Athens 139, Greece
RBL = Dr R B Lindberg, US Army Surgical Research Unit, Fort Sam, Houston, Texas 78234, USA
RHO = Dr R H Olsen, Department of Microbiology, Medical School, The University of Michigan Ann Arbor, Michigan 48104, USA
CGM = Mr C G Mason, *Biological Sciences*, Research Department, Boots Pure Drug Company Ltd, *Nottingham 56755, England*
EM = Dr Eugenia Meitert, Institutul de Microbiologie, Parazitologie si Epidemiologie 'Dr I Cantacuzino", Spl Independentei 103, Bucharest, Rumania
JDP = Dr J D Piguet, Section de bactériologie, Institut d'hygiène, 22 quai de l Ecole-de Médecine, Geneva, Switzerland
VLS = Dr Vera L Sutter, *Veterans Administration Center, Wilshire* Boulevard Los Angeles, *California* 90073, USA
HCZ – Dr H C Zanen, Gemeente Ziekenhuis, Wagnerlaan 75, Arnhem, the Netherlands

2) Typing sets

CIRL = Cross Infection Reference Laboratory During 1961–1962 36 phages were selected at CIRL. Thirteen of these phages and the PK phages except phage 6 were used at CIRL from October 1962 The set of 13 phages were sent to VLS under the designations C12–C24
EM = Meitert, reference (31)
G – Graber *et al* (21)
HCZ – Zanen (50)
HD = Hoff & Drake (23)
JDP = Refers to a set of phages 2 5, 7–8, 10–24, and 50–52
PF – Postic & Finland (41)
PK = Pavlatou & Kaklamani (40)
RBL = Lindberg *et al* (29, 30)
VLS = *Sutter et al* (46)

*) … (Bergan, 1971ab)
†) … 2B, K12A 12B, were from same

*) s = sewage, *l* = *lysogenic strain*
‖) Indicates the percentage of strains lysed among 486 strains of *P aeruginosa*

were but slight, the following phage pairs were obtained from the same aliquots 2–3, 5–6, 8–9, 28–29, 46–47, 57–58, 66–67, 100–101 (code numbers as explained in Table 1) The Sjs and Sφ indices expressing the host range similarity for these *pairs* and the per

Lytic Spectrum

The lytic spectrum of the new set inclu ding the auxiliary set ■ indicated in Table ■ This has been determined as for staphylo coccal phages (11) but even with meticulous attention to identical technique and always starting with freeze dried bacterial test strains a variation in the numerical scores from time to time have been unavoidable with pseudo monas and may amount to as much as four score increments

Plaque Morphology

The plaque morphology of the phages in cluded in the new primary and auxiliary sets appears in Fig 1

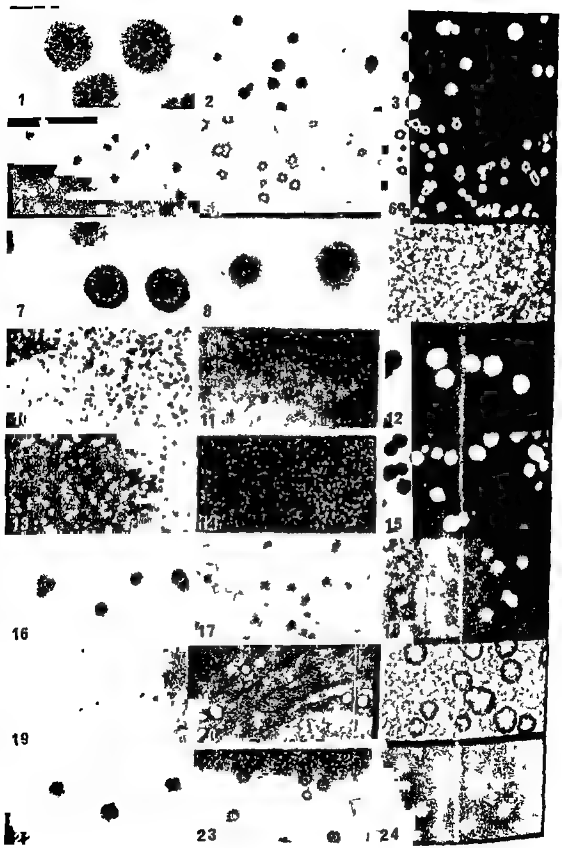
TABLE 4 *Mean Number of Positive Reactions and Mean Similarity Index Values of 27*) Clusters Re d Pair Group Average Cluster Analysis of the Trans esulting from Host Range Evaluation of 113 Bacte*

Group	Phage strains§)	Mean number of positive reactions within clusters	Mean φ
1	59 70 105	6 7	77 0
2	78	122 0	× †
3	42 43	24 0	67
4	45 46 47	11 3	66 7
5	21 49	13 5	70
6	53	4 0	×
7	97	6 0	×
8	98 99	55 5	86
9	96	29 0	×
10	89 25 24 91 100 101 111 95 92 112	19 2	68 1
11	60 71	28 5	70
12	7 20 62 84 88	89 8	68 3
13	102	69 0	×
14	90 93 75 104	109 0	64 3
15	94	35 0	Y
16	37 5 6 38 22 57 58 67 26 27 28 31 30 32 33 34 63 72 17 55 65	141 5	69 1
17	8 9 52 54 69 83 103 106 107 109	113 9	70 1
18	12 41	?3 0	89
19	1 2 3 40 68	175 0	80 9
20	80 4 15 29 23 13 16 82 10 64 65 73 56 11 18 44 48 81 86	115 0	74 8
21	14 39 35 36 61 66	132 8	76 2
22	110	43 0	×
23	50	4 0	×
24	19	2 0	×
25	51	10 0	×
26	74	28 0	×
27	76 77 108 113 79 87	34 3	70 8

* There was no level at which 25 clusters would result allowing the formation of the same number of clusters as in Table

§ Succession of numbering according to their place in the corresponding phenogram (Fig 3 reference (7))

† No similarity index possible for clusters with one member


1
2
3
7
8
11
12
15
16
17
19
23
24

DISCUSSION

Presently a long series of species is typable by phages amongst others *S aureus* (11) *Salmonella typhi* and other salmonella serotypes (3) *Escherichia coli* (37) *Mycobacterium tuberculosis* (4), *Yersinia enterocolitica* (33), and *Brucella* spp (27) Particular staphylococcal phage typing sets have been developed for bovine strains (13) and for canine strains (25) However nearly always the sets have been presented as *faits accomplis* all finished and ready for use The selection procedure as such has received only a little documented attention Some information on the selection process is available mainly for the pioneer phage typing sets the *S typhi* system of Craigie & Yen (12), and the international typing set for *S aureus* (11) The former set for Vi antigenic bacteria consisted entirely of phages belonging to the same serogroup (II) all of which were host modifications of each other They were obtained by adaptations on bacteria from the successively diminishing portion of non typable strains

Fisk (19) was the first to phage type *S aureus* His set was a more primitive system where one bacterial strain was spotted on another to detect lysogeny *Wilson & Atkinson* (48), whose set has subsequently developed into the international typing set for *S aureus* employed some phages from lysogenic strains and some phages developed in *Craigie & Yen fashion* by host modifications (38) Serological studies though later showed that some of the adapted phages were unrelated to their parent phages (38) Accordingly, these were most likely phages actually carried by the very bacterial strains on which adaptation had been attempted *Wilson & Atkinson* (48) were also influenced by *Cowan's* finding that human staphylococcal strains by serotyping

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Fig 1 Plaque morphology of bacteriophages included in the typing set for *Pseudomonas aeruginosa* The phage designations are in order of the numeration indicated in the *lower left* hand corner for each phage 73 F7 M6 Me13 113 F116 Pa3 Clc C1 C13 H249 P10 VII XVI Z2 Z3 Z19 720 21B 68 Col 11 k9 C15

The *agar layer technique* of Adams (1) has been employed The cultures were incubated at 37°C for 24 hours Picture 11 shows bea ... nt

... 22

TABLE 5 *Examples of Results Obtained by Computer Testing of Alternative Bacteriophage Typing Sets*

Phage set designation*)	Number of phages	Per cent lysed by set	Number of patterns	Mean number of reactions per plate
M	22	85 6	250	2 6
O	18	70 0	138	1 4
P	18	69 1	132	1 4
R	19	72 2	15?	1 5
T	22	81 9	232	2 4
W	20	80 7	233	2 7
AE	2?	81 1	253	2 9
ANA	19	87 9	245	2 6
ANB	19	86 4	?37	2 6
ANC	1?	86 6	232	2 6
AND	19	87 0	235	2 6
ANE (new set)	19	88 3	240	2 6
ANF	19	86 8	241	2 5
ANG	19	87 0	234	2 6
ANH	20	88 5	259	2 7

* Phage set constituents

Set M 12 17 21, 43 45 51 54 67 68 71 74 78 82 87 88 94 99 107, 104 105 110 111

Set O 12 17 21 24 43 45 51 54 60 74 78 79 89 96, 99 102 105 110

Set P 12 24 43 45 49 51 54 60 74 78 87 89 94 96 99 102 105 110

Set R 7 17 17 ... 21 [illegible]

Set T [illegible]

Set W [illegible]

Set AE ... 20 21 32 39 43 52 54 60 69 74 75 80 85 87 94 102

Sets ANA ANH have in common the phages 12 17 21 43 45 54 68 71 74 78 82 88 95 107 104 105 110 111 In addition the individual sets have ANA 36 ANB 22 ANC 56 AND 64 ANE 39 ANF 75 ANG 18 ANH 75 64

TABLE 6 *Lytic Spectrum of the New Bacteriophage Typing Set for* Pseudomonas aeruginosa.

Bacterio										Propa
phages	73	F7	M6	Me13	113	F116	Px3	C1c	C4	C13 (
73	5	5								
F7		5	2	2		2	2			2
M 6			5			5	1		1	
Me 13			2	5	5	5	1	5		5
113				4	5					
F 116			5	5	5	5		5		5
Px 3						3	5			2
C 1c							3	5	1	
C 4		1				1	3		5	
C 13	4	4	3			5	5		3	5
C 21			5	5	5	5	4	5		
H 249			3	4		3		4		3
P 10			1				5			
VII								3	4	
XVI		5	2	3				5		
Z 2	1	1	1				3		5	
Z 3				1		1				1
Z 19			2				1	5		
Z 20			5				1			
21 B			5	2			4			
Col 11			2	2					3	
68										
C 15	5		2				3	5	5	
A 9	1							5		

The reaction scores are
5 = + + reaction at RTD
4 = + + reaction at 10 × RTD
3 = + + reaction at 10^2 × RTD
2 = + + reaction at 10^3 × RTD
1 = + + reaction at 10^4 × RTD
− weak or varying reaction

were divisible into four groups Staphylococci, which had been serologically defined were used during development of the *Wilson & Atkinson* phage typing set (38)

The *Craigie & Yen* phages had narrow host ranges (38) most of the types, except type A, being characterized by reactions with one single phage occasionally with a few supplementary reactions *Wilson & Atkinson* (48) worked out their typing set under the assumption that a system similar to that for the Vi phages was achievable also in *S aureus* Such attempts, however, have always failed since a single phage adaptable in the same way as the *Craigie & Yen S typhi* phages awaits its recognition in *S aureus*

The selection of the new pseudomonas phage typing set has been achieved by the assistance of numerical allocating procedures for objective and automated comparisons of lytic spectra for the bacteriophages By cluster analysis, the phages which had related lytic spectra could be grouped together On the hand of the [Sφ] UWPGA and [Sjs] UWPGA phenograms presented previously (6, 7), various combinations of phages were chosen such that each phage represented its own phenon – of supposedly similar phages It turned out that at the phenon level where approximately 25 clusters emerged, in the [Sjs] UWPGA dendrogram one single cluster comprised an overwhelming number (69 in all) of phages and only two other clusters had more than two elements whereas in the [Sφ] UWPGA phenogram the phages were somewhat more evenly distributed among the clusters The set which ultimately was considered to best satisfy the criteria set up for a typing set was

ns

249	P10	VII	XVI	Z2	Z3	Z19	Z20	21B	Col11	68	C15	K9
									5	4	4	
2	2		3					3	1		2	
1	4	5		2	2	4	5	4		4		
3	3				1	1		5		5	2	5
5								5		5		5
5			3					5	5	5		5
4						5		1		5		5
4			3	[illegible]		3	3	5	4	3		
3		3						3			4	
5								5		5		5
5			2					5	5	5	1	4
	5	5	2				5	5	5	3		
		5	4								5	5
3		5	5	4			3	5	3	5		5
3	2			5		2		5	4	4		
5	1				5	2		5	1		1	5
1						5		2		5		
3		5			1	1	5					
						5	4	5		5		
3	4			4				5	5	5		5
5										5		
3	2	5				3	1	5	5	5	5	[illegible]
										5		5

derived from the [Sφ]UWPGA phenogram

At this point it may be mentioned that besides the inclusion of a large number of phages from previous typing sets a prerequisite for the selection of the new phage set was a valid calculation of lytic range similarity indices To achieve the latter an adequate number of reactions (here the bacterial strains) was necessary these should be independent of each other In order to obtain bacterial strains of different types approximately half of the presently used bacteria were from a wide range of foreign sources and those collected at this institute were isolated over as long as three years and came from all departments of the hospital

The advantages of such an automated procedure is that (a) a large body of data may be scanned simultaneously and that (b) an objective evaluation and comparison of the lytic spectra of the phages becomes possible Beside their indisputable value in comparing lytic spectra for phages considered during the construction of a new typing set numerical allocating procedures constitute valuable aids also when a typing set must be modified due to the inevitable changes in phage susceptibility of epidemical strains which will be the result after some time Such changes have been necessitated in the international phage typing system for staphylococci

Based on cluster analysis dendrograms various combinations of phages were selected for further investigation Based on the author's experience with one of the previous pseudomonas typing sets (30) the points considered in selecting the new typing set were (a) it should lyse as many epidemical strains as possible leaving a minimum of nontypable strains, (b) it should produce good differentiation between unrelated bacterial strains by rendering a large number of dif

ferent reaction patterns, (c) it should operate with a convenient number of reactions per plate The first and second points are self explanatory The third item has been added because the relatively large number of reactions occurring simultaneously with several previous typing sets made type pattern codes unnecessarily long When phages which lyse a large percentage of the bacteria are included, the type patterns may (falsely?) appear to be more similar than if phages with narrow lytic spectra are used Both points (b) and (c) are functions of combining phages with lytic spectra which only overlap to a modest degree This could be ascertained more efficiently by cluster analysis than if selection had been based on manual card sorting

Another aspect which one tried to consider during phage selection was concordance between phage lysis and serogroup/pyocine type of the bacteria lysed This was inspired by *Wilson & Atkinson* (48) who attempted to select phages that primarily lysed strains within one of *Cowan* s serogroups (38)

For pseudomonas, *Gould & McLeod* (20) reported that there was a connection between phage type and serogroup However, although it would be easily conceivable how identical phage receptors could be accompanied by identity in antigenic properties, examinations with the present 113 phages and 486 pseudomonas strains of varied origin, failed to substantiate their findings (5) Subsequent studies on staphylococci have shown that the correlation between phage susceptibility and the serogroup there also is not as good as was originally presumed It is possible that the reported relationship between lysis and serology (20) has been a consequence of having used a sample of bacterial strains which was either too small, or fairly homogeneous regarding phage susceptibility That would result if strains were collected either over a short time span and/or from only a few sources

During the development of the new phage set, it was found preferable, admittedly from purely subjective deliberation, to operate with a typing set of 19 phages for a primary typing set using phages which mostly had 10–15 per cent lysis, and an auxiliary set of 5 relatively avid phages lysing as many as possible of the strains which were primarily non typable It was found convenient to include the auxiliary phages on the first typing plate, thus left one position in the 5 × 5 pattern of phage drops unoccupied such that the orientation on the plates was simple If all these 24 phages were included in one single set the same pseudomonas strains would be typable, but the type pattern code would be longer, less practical to handle, and thus violate the prerequisite for few lytic reactions per plate It is preferable to operate with an auxiliary set rather than to retype non typable strains with more concentrated phage suspensions as is done with the staphylococci, due to the possibility in pseudomonas of interference from pyocines Using concentrated phage suspensions might result in a combined phage and pyocine typing technique akin to the fingerprinting method (17) or the *Osman* pyocine typing procedure (36)

The list of *References* is shared with part 2 in this series and follows on pages 200–201

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A NEW BACTERIOPHAGE TYPING SET FOR *PSEUDOMONAS AERUGINOSA*

2 *Characterization and Comparisons of New and Previous Typing Sets*

TOM BERGAN

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A new phage typing set for *Pseudomonas aeruginosa* has been established on the basis of all internationally available typing sets and a few additional phages The present paper describes some of the important characteristics of the new phage typing set compared to previous sets In terms of the relevant parameters the new phage typing set appears to constitute an improvements compared to previous alternatives

For pseudomonas, several phage typing sets have been introduced On the basis of some of these, a new typing set has been constructed (8) It is the purpose of the present report to compare this to previous sets and document some of its advantages

MATERIALS AND METHODS

Materials and methods and terminology have been as presented previously (6 8)

RESULTS

Similarity Matrix for the New Phage Typing Set

The similarity matrices for the Jaccard Sneath (S_{JS}) and the transformed Yule correlation coefficient ($S\varphi$) for the new primary and auxiliary phage typing sets are presented in Table 3

Propagation Titers

The significance of selecting phages which were attainable in high titers and which remained stable under usual storage conditions for long periods is obvious The propagation titers reached and the drop in phage titers after approximately two years (21–24 months) storage in propagation broth at 4° C are indicated in Table 2 The largest drop in infectivity was found for C13 and C21

Similarity Matrices for Previous Phage Typing Sets

From the phages that have been available for this study, the similarity matrices for the previous pseudomonas phage typing sets are given to the extent that the phages have been available for study in Tables 3–7 The sets of (a) *Hoff & Drake* (23), (b) *Graber et al* (21), (c) *Piguet*, and (d) the Cross-Infection Reference Laboratory (see Table 1, reference (8)) are roughly discernible from Table 3

Comparisons of Previous and New Phage Typing Sets

In Table 8 are recorded both the percentages of pseudomonas strains typable as re-

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TABLE 1 *Similarity Matrices According to the Jaccard Sneath Similarity Index (S_{JS}) and a Transformed Yule Correlation Coefficient (S_{φ}) of Host Ranges for a Bacteriophage Typing Set for* Pseudomonas aeruginosa *Selected from 113 Internationally Available Phages Mostly from Previous Typing Sets Which Were Tested on 486 Strains of* Pseudomonas aeruginosa *The Code to the Phage Designations Is Given in Table 2*

S_{JS}

	12	17	21	39	43	45	54	68	71	74	7[illegible]	82	88	95	102	104	105	110	111	6	11	22	64	75
12	×																							
17	2	×																						
21	2	[illegible]	×																					
39	5	3	[illegible]	×																				
43	0	11	7	[illegible]	×																			
45	2	0	11	[illegible]	3	×																		
54	4	19	3	3	17	2	×																	
68	12	2	7	29	[illegible]	7	5	×																
71	0	7	6	15	7	2	6	12	×															
74	[illegible]	2	4	10	10	16	3	2	5	×														
78	7	7	3	12	4	2	9	14	8	3	×													
82	5	3	3	21	13	6	8	23	9	4	12	×												
88	4	7	4	15	9	3	13	13	15	[illegible]	16	16	×											
95	0	12	6	10	4	0	7	[illegible]	19	9	10	4	6	×										
102	4	4	7	19	13	7	9	12	12	10	14	10	19	9	×									
104	2	14	2	17	15	3	19	13	19	5	18	18	23	10	19	×								
105	3	0	3	3	6	3	0	1	4	5	4	3	2	2	6	3	×							
110	2	1	3	18	5	7	6	5	13	9	6	8	13	11	15	14	5	×						
111	0	2	7	8	3	2	2	4	5	6	5	7	7	25	6	7	5	4	×					
[illegible]	3	11	5	25	12	7	20	20	9	7	17	15	14	10	14	24	2	12	9	×				
11	3	6	4	25	14	9	9	27	13	10	17	41	16	8	14	19	3	10	6	30	×			
22	5	15	4	17	17	3	30	18	10	6	17	21	19	7	10	27	1	11	3	29	[illegible]	×		
64	5	3	7	23	13	9	8	25	11	8	8	44	14	4	15	18	4	11	4	18	42	20	×	
75	1	8	2	12	9	5	21	10	14	7	15	15	28	9	19	30	2	9	4	16	18	16	11	×

ported originally for the previous typing sets, and the way these sets acted on the present collection of 486 pseudomonas strains, in terms of (a) the percentage of strains typed, (b) number of phage patterns observed, and (c) *mean number of reactions per plate It is demonstrated that the new typing set renders a high percentage of typable strains,* a comparatively good differentiation, and relatively few lytic reactions per plate

Similarities of Phages Obtained from Different Sources but with a Common Origin

Table 9 shows a remarkably low degree of similarity for phages that were originally the same, but have been obtained from different sources where they have been used in typing sets

DISCUSSION

The background for the present selection of yet another phage typing set for *P aeruginosa* was that an improvement of previous sets was felt necessary (*M T Parker* and *E Asheshov, personal communication,* 1967) *for instance, in terms of the percentage of lysis Table 8 shows that the new typing set* compares rather favourably with previous *sets* Lysing 95 5 per cent when the auxiliary set is included, the new alternative lyses a higher portion than any previous set, except a pilot study set (22), which has not been available for the present investigation Phages with a wide host range being of little use for typing (38), this set (22) however, is no real alternative since 6 of its 10 phages lyse more than 60 per cent of the bacteria From the findings in Table 8, it appears that the percentage lysed among the 486 pseudomonas

TABLE 1 *continued*

Sp	12	17	21	39	43	45	54	68	71	74	78	82	88	95	102	104	105	110	111	6	11	22	64	75
12	×																							
17	49	×																						
21	50	47	×																					
39	51	47	56	×																				
43	47	56	55	53	×																			
45	50	47	58	56	59	×																		
54	50	64	50	41	61	48	×																	
68	62	45	56	63	52	55	42	×																
71	47	53	54	61	53	49	51	58	×															
74	49	48	52	57	56	62	48	57	52	×														
78	53	52	49	49	46	47	48	49	53	48	×													
82	51	47	49	58	57	53	48	59	54	49	49	×												
88	50	52	57	55	53	49	54	51	61	52	55	56	×											
95	47	58	53	55	50	47	52	50	64	55	55	48	50	×										
102	51	49	55	59	58	54	50	52	57	56	54	51	60	55	×									
104	46	60	49	54	59	49	58	49	65	50	54	56	62	55	59	×								
105	51	48	51	51	52	51	48	47	52	53	52	50	49	50	53	51	×							
110	48	47	51	61	61	54	50	47	59	55	49	51	57	57	59	58	54	×						
111	47	49	53	55	56	50	47	49	52	53	51	53	54	68	52	53	53	56	×					
6	48	59	52	58	57	55	58	49	55	53	48	48	51	56	52	57	48	56	58	×				
11	47	50	51	59	53	58	45	58	60	58	50	73	54	53	53	54	50	53	52	56	×			
22	51	61	50	53	61	48	67	52	55	52	52	58	57	52	50	62	47	55	48	60	58	×		
64	52	47	55	60	58	57	48	60	56	54	44	75	53	48	55	55	52	54	49	50	73	[illegible]	×	
75	46	53	48	51	53	51	60	47	59	53	53	54	66	53	60	66	49	53	49	51	54	54	49	×

TABLE 2 *Bacteriophage Titres at the Time of Propagation and after 21 24 Months Storage in Propagation Broth at 4° C*

Bacteriophage	Phage code	Titer at propagation	Titer after storage	Bacteriophage	Phage code	Titer at propagation	Titer after storage
73	12	6 0 10^{9}	4 7 10^{9}	P10	88	6 7 10	4 7 10^{6}
F7	17	2 8 10^{9}	3 6 10^{9}	M11	95	3 8 10^{8}	1 1 10^{4}
M6	21	2 2 10^{9}	4 8 10^{7}	M11	102	3 0 10^{7}	9 1 10
Me13	39	5 2 10^{9}	7 2 10^{8}	22	104	8 9 10^{9}	2 7 10^{5}
M3	43	3 10^{10}	6 9 10^{4}	Z3	105	4 7 10^{10}	3 9 10^{9}
F116	45	1 5 10^{11}	7 1 10^{9}	Z19	110	2 0 10^{9}	4 2 10^{7}
Pe3	54	6 8 10^{5}	2 1 10^{6}	Z20	111	1 5 10^{5}	3 7 10^{7}
Clc	68	6 1 10^{9}	4 8 10^{7}	21B	6	2 7 10^{9}	4 5 10^{8}
C4	71	5 0 10^{6}	5 0 10^{6}	68	11	5 2 10^{8}	4 5 10^{6}
Uf5	74	1 6 10^{4}	7 1 10^{5}	Col 11	22	1 0 10^{7}	3 0 10^{4}
C21	78	6 7 10^{7}	2 3 10^{5}	K9	64	2 5 10^{9}	6 2 10^{7}
H249	82	1 4 10^{8}	5 2 10^{8}	C15	75	6 2 10^{7}	7 8 10^{5}

strains used here for all previous typing sets as they were published or with subsequent modifications was lower than the percentage of lysis reported originally The *Lindberg et al* set (30) which initially produced 92 per cent lysis only reacted with 86 per cent of the 486 pseudomonas strains The *Meitert* (31) set dropped from 92 to 74 per cent The results obtained with the *Sutter et al* set (46) are remarkable since exactly 86 4 per cent lysis was obtained both originally and with the present collection of bacterial strains

TABLE 3 *Similarity Matrix According to the* ... *ed Yule Correlation Coefficient (Sφ) of Host Ra*... *Set Supplemented by Bacteriophages 22–24, 50–52* ... aeruginosa *Code to Phage Designations Apparent from Table 1, Reference (8)*

Sjs

	1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	50	51	52	40	41	42	43
1	×																											
[illegible]	51	×																										
4	18	18	×																									
5	35	20	14	×																								
7	21	14	15	17	×																							
[illegible]	32	12	7	21	13	×																						
10	26	34	29	22	13	12	×																					
11	28	33	26	32	15	9	52	×																				
12	9	11	[illegible]	1	4	7	[illegible]	3	×																			
13	22	30	31	23	15	10	50	48	6	×																		
14	31	27	15	27	17	14	23	26	15	22	×																	
15	18	24	24	16	18	9	36	35	2	34	11	×																
16	34	39	23	24	14	13	58	53	5	50	23	33	×															
17	12	[illegible]	0	12	16	18	4	6	2	6	17	4	6	×														
18	26	33	36	21	15	11	59	63	3	59	23	40	54	3	×													
19	1	1	2	1	2	0	1	1	0	0	1	0	1	[illegible]	1	×												
20	20	20	21	13	36	6	12	15	8	17	17	18	15	4	17	2	×											
21	4	5	5	5	4	1	6	4	2	4	5	6	4	0	4	0	7	×										
22	43	22	16	29	28	33	24	23	5	19	25	20	25	15	23	1	22	4	×									
23	31	37	23	23	21	16	48	50	4	41	27	34	52	5	55	0	18	5	24	×								
24	2	2	2	5	3	4	3	2	0	10	5	1	3	2	3	0	3	0	2	2	×							
50	2	0	0	2	3	1	1	2	0	1	1	4	2	6	1	0	4	0	2	1	[illegible]	×						
51	4	4	9	3	8	2	4	3	0	4	4	2	3	2	4	9	9	0	4	2	5	8	×					
52	27	13	4	25	12	39	13	11	3	12	12	15	19	15	12	0	6	1	29	19	2	1	1	×				
40	52	63	16	24	20	16	24	27	12	24	31	22	32	7	25	1	21	4	26	31	2	1	3	15	×			
41	9	10	4	2	3	6	4	2	64	3	12	3	3	0	2	0	7	3	6	2	0	0	0	2	12	×		
42	2	1	10	2	2	1	2	2	3	3	2	2	2	0	3	0	0	4	1	1	0	0	0	1	2	4	×	
43	13	6	14	13	10	17	13	14	0	12	4	16	12	11	15	0	6	7	17	12	0	7	2	16	10	2	14	×

Even if the new primary set alone were considered, it exhibited a percentage of lysis which being 88 3 compares favourably with the previously developed typing systems This percentage, by far, exceeds what has been found in other species In the original staphylococcal phage typing set of *Wilson & Atkinson* (48), with 22 phages, 83 per cent were typable With the international staphylococcal phage typing set (11), roughly 70 per cent are typable at the routine test dilution (RTD) (38) In a Norwegian collection, only 48 per cent of the staphylococci were typable (14) In *E coli*, 34–82 per cent have been typable (37)

It has been gratifying to see that the new set has a high percentage of typable strains also on collections of pseudomonas unrelated to that on which it was developed Thus, on a collection of 349 strains derived mostly from animals, only 7 4 per cent were non typable (9) Of 302 Polish strains, 16 4 per cent were not typable *(26) compared to* 4 5 per cent for the present 486 strains

Together with the percentage of bacteria lysed by a set, the number of reactions occurring on each plate is to be considered *It* is more convenient to operate with fewer reactions The new typing set alternative has a mean of 2 6 reactions per plate when the auxiliary set is not counted whereas most previous sets have close to 4 reactions (Table 8) Since these other sets have a lower portion of typable strains, the pattern codes are in reality far longer *Zanen's* set (50) has a mean of 3 1 reactions per plate, but lyses only

TABLE 3 *continued*

Sφ	1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	50	51	52	40	41	42	43
1	×																											
2	74	×																										
4	62	62	×																									
5	61	45	57	×																								
7	62	54	58	58	×																							
8	67	47	50	57	55	×																						
10	57	65	71	53	54	49	×																					
11	56	60	70	58	56	44	78	×																				
12	57	62	52	44	50	54	52	47	×																			
13	56	63	72	57	56	49	78	77	[illegible]	×																		
14	62	59	58	59	58	51	58	58	64	58	×																	
15	55	62	66	53	59	49	71	71	48	70	50	×																
16	61	65	67	51	55	49	82	76	51	78	56	70	×															
17	60	44	46	60	61	64	48	[illegible]	49	51	64	49	51	×														
18	58	65	76	53	56	49	83	85	48	83	58	74	80	47	×													
19	51	51	54	51	54	48	52	51	49	48	52	49	51	49	52	×												
20	63	63	64	55	74	48	54	57	55	59	59	60	57	49	59	54	×											
21	50	52	52	54	51	47	54	51	50	51	53	54	50	47	52	49	55	×										
22	72	54	59	60	68	68	58	55	51	55	59	59	51	61	58	52	64	50	×									
23	61	65	66	53	62	53	76	75	50	72	60	70	77	49	80	48	61	53	58	×								
24	49	47	49	55	51	53	51	48	48	62	54	48	51	50	51	49	51	48	49	49	×							
50	54	46	48	56	55	51	50	54	49	50	50	57	54	58	50	50	56	49	55	52	49	×						
51	56	55	60	53	58	50	54	54	48	55	54	51	52	51	54	61	59	49	54	50	53	57	×					
52	58	43	43	56	53	72	47	42	47	47	46	54	51	61	47	48	47	43	63	52	49	52	49	×				
40	75	82	[illegible]	50	62	52	56	56	63	58	63	60	60	52	58	51	64	51	59	61	49	51	54	46	×			
41	[illegible]	62	51	45	49	52	50	46	89	49	62	49	48	47	47	49	54	50	53	47	48	49	48	47	63	×		
42	51	49	62	50	50	49	52	53	53	53	50	52	51	48	54	50	48	53	48	50	49	49	49	48	51	53	×	
43	[illegible]	48	58	58	54	61	58	59	47	56	47	60	57	56	59	49	51	55	61	56	47	61	50	61	54	48	67	×

¾ of the epidemiological strains, the *Pavlatou & Kaklamani* set (40) (abbreviated by 3 phages compared to the published set) which in Table [illegible] operates with only 2 reactions per plate, showed only 70 per cent lysis. In addition the number of phage patterns resulting with this abbreviated set (40) was exceptionally low, 78 as compared to the 240 patterns for the new (primary) typing set. Although in a few other previous typing sets more patterns resulted than in the new set, these [illegible] evidenced by current findings were less favourable in terms of per cent lysis and the mean number of reactions per plate. Still, one should not be mislead to think that the sheer number of different patterns in itself is a sufficient parameter, since it could be that a set with fewer, but stabler patterns were more suitable.

Connected with both the degree of differentiation and the number of reactions per plate is the similarity in lytic spectra for the phages included in a set. In this investigation, we are fortunate enough to have available objective measures for the interrelationships between phage lytic spectra (Tables 1, 3–7). The low similarity index values of S_{JG} and $S\varphi$ for the new set (Table 1) are in contrast to the situation for the other sets (Tables 3–7), where a considerable portion of the phages exhibit higher similarities. It is noteworthy that the lytic ranges of the *Meitert* phages (31) were so much alike that most of them actually emerged in the same cluster after clustering analysis (6, 7), regardless which similarity statistic was employed.

Another interesting aspect discernible from this investigation was the considerable variation in lytic activity which arose when pseudomonas typing bacteriophages were propa-

TABLE 4 *Similarity Matrix According to the Jaccard-Sneath Similarity Index (S_{JS}) and a Transformed Yule Correlation Coefficient ($S\varphi$) of Host Ranges on 486 Strains of* Pseudomonas aeruginosa *for the* Meitert *(31) Bacteriophage Typing Set Code to Phage Designations Apparent from Table 1, Reference (8)*

S_{JS}

	26	27	28	30	31	32	33	34	35	36	37	38	39
26	×												
27	76	×											
28	75	84	×										
30	56	62	63	×									
31	62	72	71	62	×								
32	47	51	54	71	50	×							
33	51	55	55	69	54	67	×						
34	45	49	49	61	53	60	70	×					
35	15	20	20	27	21	24	28	28	×				
36	14	19	19	25	22	21	26	30	72	×			
37	33	40	38	41	38	34	42	42	28	24	×		
38	28	33	32	37	32	30	37	37	30	27	57	×	
39	14	22	21	26	20	21	24	26	53	53	24	21	×

$S\varphi$

	26	27	28	30	31	32	33	34	35	36	37	38	39
26	×												
27	91	×											
28	90	94	×										
30	61	64	63	×									
31	81	83	84	83	×								
32	76	78	80	89	77	×							
33	79	80	80	87	79	87	×						
34	74	75	76	82	77	83	88	×					
35	51	55	55	60	54	59	62	60	×				
36	50	53	54	58	55	56	60	62	89	×			
37	65	68	67	68	65	65	71	68	60	56	×		
38	61	64	64	66	62	63	67	66	62	60	79	×	
39	51	57	56	60	54	57	60	60	80	80	57	56	×

1..

' ccard Sneath Similarity Index (S_{JS}) and a Transformed on 486 Strains of Pseudomonas aeruginosa *for the Pa Set Code to Phage Designations Apparent from Table 1, Reference (8)*

S_{JS}

	57	59	60	61	62	63	64	65	66
57	×								
59	0	×							
60	7	0	×						
61	18	0	6	×					
62	29	0	10	23	×				
63	45	0	6	30	35	×			
64	15	1	5	27	21	22	×		
65	13	0	6	24	22	21	79	×	
66	22	0	5	79	24	31	25	21	×

$S\varphi$

	57	59	60	61	62	63	64	65	66
57	×								
59	48	×							
60	53	49	×						
61	52	47	52	×					
62	65	48	57	58	×				
63	74	47	53	60	69				
64	51	51	51	61	58	56	×		
65	50	51	52	59	60	55	92	×	
66	55	55	51	91	60	61	60	56	×

TABLE 6 *Similarity Matrices According to the Jaccard-Sneath Similarity Index (S_{JS}) and a Transformed Yule Correlation Coefficient (S_Y) of Host Ranges on 486 Strains of* Pseudomonas aeruginosa *for the* Sutter et al (46) *Bacteriophage Typing Set Supplemented with the Bacteriophages 20–82 Code to Phage Designations Apparent from Table 1. Reference (8)*

S_{JS}

	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
68	×																					
69	3	×																				
70	0	0	×																			
71	12	3	0	×																		
72	25	31	0	12	×																	
73	27	9	1	12	24	×																
74	10	2	0	5	9	8	×															
75	10	19	0	14	21	15	7	×														
76	6	3	0	5	10	14	18	6	×													
77	7	1	0	5	11	11	15	7	30	×												
78	14	8	0	8	15	14	3	15	6	3	×											
79	5	1	0	11	12	12	14	7	33	24	11	×										
80	13	14	0	9	14	30	12	17	12	6	8	8	×									
81	30	9	0	15	30	51	8	21	12	11	17	11	32	×								
82	23	7	0	9	23	41	4	15	10	12	12	9	29	52	×							
83	10	31	0	5	36	16	3	22	3	5	12	2	19	17	14	×						
84	27	14	0	16	34	21	9	26	8	15	23	13	14	22	16	21	×					
85	12	32	0	11	33	15	8	17	6	8	9	7	22	17	12	31	21	×				
86	30	7	0	16	21	44	11	15	8	11	10	4	31	50	44	12	20	17	×			
87	4	4	0	19	14	10	12	12	27	33	8	43	6	12	8	6	13	10	7	×		
88	13	10	0	15	17	15	6	28	5	11	16	10	12	18	16	20	32	12	16	11	×	
89	5	1	0	2	5	3	7	4	2	14	4	2	3	5	5	3	7	2	7	5	9	×

S_Y

	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89
68	×																					
69	39	×																				
70	48	49	×																			
71	58	47	49	×																		
72	57	68	48	58	×																	
73	61	47	52	58	58	×																
74	57	47	49	52	56	54	×															
75	47	59	48	59	57	52	53	×														
76	51	47	49	51	56	61	63	51	×													
77	34	46	49	52	60	59	61	54	71	×												
78	49	46	48	53	49	49	48	53	51	47	×											
79	49	45	49	57	59	58	60	53	73	68	57	×										
80	56	56	49	55	54	69	58	58	57	52	49	54	×									
81	62	46	48	62	61	78	55	58	59	59	52	58	71	×								
82	59	47	48	54	59	75	49	54	56	59	49	55	68	80	×							
83	44	81	48	30	68	52	48	59	46	50	47	46	59	52	51	×						
84	61	53	48	63	66	57	56	63	54	63	58	60	55	56	53	57	×					
85	49	69	48	50	68	53	34	56	51	49	47	52	62	54	51	66	58	×				
86	63	45	48	62	57	76	57	54	54	58	47	49	69	79	75	49	57	55	×			
87	48	49	49	64	62	56	58	58	69	74	53	79	51	59	54	50	59	56	52	×		
88	51	51	49	61	55	54	52	66	50	57	55	56	54	56	56	50	69	52	56	57	×	
89	53	47	49	50	54	50	55	51	50	61	51	50	50	54	54	50	57	48	55	52	58	×

TABLE 7 *Similarity Matrices According to t... ed Yule Correlation Coefficient (Sφ) of Host Zanen (50) Bacteriophage Typing Set Code* ... *(8)*

S_{JS}

	90	91	92	93	94	95	96	97	98	99	100	102	103	104	105	106	107	108	109	110	111	112	113
90	×																						
91	[illegible]	×																					
92	[illegible]	24	×																				
93	34	5	2	×																			
94	10	12	5	12	×																		
95	22	21	22	12	11	×																	
96	16	18	15	13	12	16	×																
97	5	6	23	1	5	8	3	×															
98	14	2	7	15	6	13	5	9	×														
99	15	3	3	20	12	5	10	5	61	×													
100	12	43	23	7	19	24	24	4	4	5	×												
102	15	1	4	24	11	5	10	3	13	19	5	×											
103	16	[illegible]	1	20	10	5	3	0	5	9	3	12	×										
104	24	3	2	35	16	10	10	1	16	18	6	19	20	×									
105	2	4	4	4	4	2	2	5	3	3	3	6	8	3	×								
106	21	2	1	25	9	7	6	1	7	11	4	15	62	24	7	×							
107	23	1	1	27	7	5	6	1	10	15	3	16	55	29	8	69	×						
108	10	1	1	13	13	11	2	2	8	12	5	21	19	18	12	19	17	×					
109	20	1	1	27	9	7	7	0	7	10	2	14	32	27	5	65	60	20	×				
110	11	6	6	11	11	11	7	4	12	13	10	15	10	14	5	12	11	12	11	×			
111	10	[illegible]	25	6	7	25	15	7	10	5	38	6	3	7	5	4	3	4	4	10	×		
112	10	14	35	3	6	19	11	13	7	3	23	6	8	4	13	7	5	10	6	7	[illegible]	×	
113	6	2	0	7	10	8	2	3	2	6	4	1	9	10	2	11	9	31	10	9	4	4	×

$S\varphi$

	90	91	92	93	94	95	96	97	98	99	100	102	103	104	105	106	107	108	109	110	111	112	113
90	×																						
91	60	×																					
92	60	68	×																				
93	70	57	50	×																			
94	56	61	54	57	×																		
95	66	69	70	58	57	×																	
96	62	66	63	61	58	62	×																
97	58	55	69	50	56	59	53	×															
98	56	50	57	57	52	58	51	63															
99	57	51	52	62	57	55	56	57	86	×													
100	60	82	69	54	64	68	68	53	51	52	×												
102	57	49	53	64	56	55	[illegible]	53	56	61	51	×											
103	55	49	48	55	55	50	48	47	47	51	49	53	×										
104	62	51	50	68	61	55	56	49	58	59	53	59	58	×									
105	49	53	53	53	52	50	50	54	51	50	51	55	59	51	×								
106	59	50	47	57	54	51	51	49	49	53	50	54	85	60	58	×							
107	61	48	48	57	51	48	51	48	52	56	48	55	82	63	60	87	×						
108	53	49	50	54	58	56	48	51	52	55	52	63	61	59	61	60	59	×					
109	58	48	47	58	[illegible]	52	53	46	48	50	47	59	80	61	54	85	82	61	×				
110	55	53	55	55	57	57	54	55	56	58	57	59	54	58	54	56	55	57	55	×			
111	57	73	71	53	54	68	61	57	56	51	77	52	49	53	53	49	47	50	49	56	×		
112	57	62	77	49	53	65	58	63	54	49	67	53	56	51	60	55	52	57	51	54	70	×	
113	51	51	48	53	56	55	48	52	48	52	51	57	55	56	50	57	54	72	56	55	51	51	×

TABLE 8 *Characteristics of Previous and the New Bacteriophage Typing Sets for* Pseudomonas aeruginosa

Set reference, year	Phages included*)	In current use	Percentage of typable strains seen by original authors	Phages available for present study	Performance on own collection of 486 strains		
					Per cent typed	Number of phage patterns	Mean number of reactions per plate
Potel (42), 1957	Seven	No	?	No			
Hoff & Drake (23), 1960	2, 3, 7, 9, 11, 16, 21, 27, 31, 44, 68, 73, 91, 95, 109, 111, 113, 116, 249	No	70.0	Yes, except 9, 27, 91, 111			
Gould & McLeod (20), 1960	B, D, G, H, I, J, K, N, O, Q, R, S, T, W, X, Z	No	92.0	No			
Meitert & Meitert (32), 1961	A, B, C, D, E, F, G, H, I, J, K, L, M	No	66.4	No			
Pavlatou & Kaklamani (40), 1961	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	No?	79.3	Yes, except 11	69.6**)	78	2.0
Postic & Finland (41), 1961	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13	No	88.8	Yes, except 1, 3, 5, 11, 13			
Graber et al (21), 1962	2, 3, 7, 9, 11, 16, 21, 24, 27, 31, 44, 68, 73, 91, 95, 109, 111, 113, 116, 352, 1214	No	92.0	Yes, except 9, 27, 91, 111			
Lindberg et al (29, 30), 1963, 1964	2, 7, 16, 21, 31, 44, 68, 73, 109, 119x, 352, 1214, F7, F8, F10, M4, M6 †)	Yes	92.0	Yes	86.2	224	3.9

TABLE 8 *continued*

Set reference, year	Phages included*)	In current use	Percentage of typable strains seen by original authors	Phages available for present study	Performance on own collection of 486 strains: Per cent typed	Number of phage patterns	Mean number of reactions per plate
Zanen (30), 1963	I, IV, V, VII, IX, X, XI, XII, VI, III, XVI, XIII, 1, 2, 3, 6, 9, 16, 18, 19, 20, 21, 22	No	?	Yes	74 7	219	3 1
Meitert (31), 1965	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13	Yes	92 5	Yes	73 5	158	4 0
Sutter et al (46) 1965	C1, C3, C4, C6, C7, C9, C13, C16, C19, C21, C22, P2, P6, P7, P8, P9, P10, P12	Yes?	86 4	Yes, except C6	[illegible] 4§)	278	3 9
Grun et al (22) 1967	PsPL, PsPL1, PsP60, PsPb, PsD1, PsD4 PsD5 PsD6, PsD9, PsD10	?	97 7	No			
Modified Lindberg et al sets (unpublished)	A Includes all Lindberg et al phages but no 2 but with Col 11, Col 18, and Col 21 added	Yes	–	Yes	88 1	278	4 5
	B Includes above set plus phages 103g, 176p,188/1	Yes	–	Yes	90 1	298	4 6
New set	Primary set see text Auxiliary set see text	Yes Yes	95 5	–	88 3	240	2 6

*) Designation of the authors Code to phage designations relative to that indicated Tables 1 and 3–7 apparent from Table 1, reference (8)
**) Calculated without the phages nos 1, 7, 11 Phages 1 and 7 available for *Sutter*, see Table 1 reference (8) and reference (46)
$) Set indicated as received from Dr *R B Lindberg* October, 1966 The 'Progress Report" for private circulation, reference (30), did not include the phages nos 2, F7, F10 in the phage typing set.
§) Calculated for an improved set received from Dr *V L Sutter* December, 1967, which had C6 excluded, H95, H116, H249 added, and C1c and C1t substituted for phage C1

TABLE 9 *Similarities Expressed in the Jaccard Sneath (S_{JS}) and the Transformed Yule Correlation Coefficient (S_{Q}) for Typing Bacteriophages with a Presumed Common Origin as Evidenced by Their Lytic Activity on 486 Strains of* Pseudomonas aeruginosa

Phage number*)	S_{JS}	S_{Q}
23 109	27	59
42 80	6	58
57 104	27	62
59 70–105	67–20–13**)	91 72–68
60– 71	27	70
6? 106	20	56
64– 73–107	62–19–25	85–54 58
76 108	27	70
77 110	23	67
78–112	4	51
79–113	33	73

*) The same numbers as employed previously (Table 1, reference (8) and in references (6, 7))

**) Indicated in sucession according to increases in the sums of the operational numerical units

Samples of the propagated phages have not been sent back to their original sources such a control could only have been carried out on two previous sets since most workers that would have been involved are no longer active in this field

gated by different competent workers Several of the typing sets had been distributed to other centers from which they were obtained for the present investigation The similarity indices between phages supposedly of identical origin (Table 9) showed considerable differences in lytic activities It would have been advantageous to have had the sources of the phages control the identity and lytic spectrum of the last propagated phages against their own batches, this control was not done for any of the preparations, since some of the phage sources were no longer involved in pseudomonas phage typing

Differences in lytic activity for some of the phages may be the result of such changes in lytic spectrum but may also be a function of differences in the bacterial populations tested For instance the *Lindberg et al* phage number 2 in my hands showed 37 per cent lysis (Table 1 reference (8)), whereas ca 47 per cent was found by others (44) Similarly, the percentages for phage number 119x were 26 and 37, for phage number 352 were 16 and 45, and for phage number F7 were 7 and 12 per cent respectively (8, 44) *Sutter et al*'s phage number C7 presently lysed 32 per cent of the bacteria, whereas in *Sutter*'s own experience it lysed only 16 per cent (*Sutter*, personal communication, 1971)

Some of the previous sets apparently have only been intended as demonstrations of the possibility of pseudomonas phage typing (20, 22, 23, 41) It [illegible] noteworthy that the phage [illegible]

modifications, or the *Meitert* set (31), which appear to be the only two sets in current use The *Lindberg* set (29, 30) has become the most widely distributed of all other sets (Great Britain, Denmark, France, Norway, Sweden, Switzerland and the USA) The impression of the present author is that the *Sutter et al* set would have been the one preferred out of all previous sets

In the previous typing sets for pseudomonas, types have either been described by numbers or letters each representing a separate phage pattern (20, 31, 40), similar to that which has been done for the salmonellas or by the pattern codes as has been done with the international phage typing set for staphylococci (3, 12, 38) Initially, one recognized a number of defined 'phage types' for the staphylococci Now, one has preferred to recognize pattern reactions because this allows very fine subdivisions, which is an advantage in epidemiological situations where numerous different strains must be differentiated (38) Similarly, pattern reactions are recorded with the present typing set for pseudomonas

Phage typing of pseudomonas has been done previously also by registrations of lysogeny (17, 18, 24 32 49) This might seem like a reasonable approach, since 70–100 per cent of pseudomonas strains have been found to be lysogenic (2 18, 39 47) *Meitert* & *Meitert* (32) found 89 per cent of 109 strains to be lysogenic, but only 70 per cent suscep-

tible to the phage set tested at the time Registering lysogeny has its disadvantages One bacterial strain may contain as many as 8 or more different bacteriophages as evidenced by physical, cultural, and serological procedures (43) Lysogeny may exist in a strain, but evade recognition *Farmer & Herman*'s work (17) showed that pyocine production may interfere with growth "inhibiting' reactions caused by bacteriophages

The presently proposed phage typing alternative for *P aeruginosa* by the parameters studied appears to be a better alternative than the phage typing sets developed previously However, first of all a phage typing set requires epidemiological evaluation To meet this, the new phage typing set is presently under evaluation on the hand of a large Polish hospital study (28) where the results of phage typing are compared with pyocine typing and serogrouping

Secondly, the phage typing set must perform well also in other locations and in the hands of others It is hoped that the set will work well also in other situations, since the bacterial strains on which the lytic spectra of the phages were compared came from world wide sources

The new phage typing set should provide a tool for better differentiation between epidemiological strains A wider distribution of the set would make international comparisons of pseudomonas phage typing from one center to another more readily feasible Studies to be reported later will show that the new set performs well also on animal pseudomonas strains (9) and will deal with some aspects of the variation found in phage type pattern (9, 10)

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ADSORPTION OF BILE ACIDS TO INTESTINAL MICROORGANISMS

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The transformations of chenodeoxycholic and lithocholic acid 24-^{14}C were studied in anaerobic cultures from dilution steps of intestinal content from the small intestine, cecum and feces from rats The distribution of metabolites between supernatant and sediment after centrifugation of the cultures was determined Lithocholic acid and its main metabolites 3β hydroxy-5β-cholanoic acid and 3-keto-5β-cholanoic acid, were partly adsorbed to the microorganisms in the cultures In contrast, chenodeoxycholic acid and 3α-hydroxy-7-keto-5β-cholanoic acid were not found to be adsorbed to intestinal microorganisms

Bile acids are excreted into the bile conjugated with the amino acids taurine or glycine In the intestinal tract the hydroxyl groups and the peptide bond are attacked by microbial enzymes The metabolites formed are to varying degrees absorbed and further transformed by liver enzymes prior to reexcretion in the bile, (for a review, see 1)

The main function of bile acids in the small intestine is related to their ability to form polymolecular aggregates, (termed micelles) with water-insoluble lipids such as monoglycerides, fatty acids, cholesterol and fat soluble vitamins The micellar properties of the bile acids are related to the numbers and sites of the hydrophilic groups present (for a review, see 5)

Both the micelle formation as well as absorption and elimination of the bile acids in the intestine seem to be dependent on the chemical nature of the bile acid present This in turn, depends upon the action of the microbial enzymes on the bile acids at various levels in the intestinal tract The two main microbial reactions are the splitting of the conjugates giving free bile acids, and the removal of the 7α hydroxyl group of cholic and chenodeoxycholic acid leading to the formation of deoxycholic acid and lithocholic acid respectively Deconjugating microorganisms are present in large numbers in cecum contents and feces from rats (7,8) They are also present, although in varying numbers, in ileum contents Removal of the hydroxyl group has been found to take place in anaerobic cultures from the lower dilution steps of cecum contents and feces in the rat (7) A 7α-dehydroxylation could not be demonstrated in ileum contents or in the higher dilution steps of cecum contents and feces of the same rats This might be due to either unfavourable culture conditions for 7α dehydroxylating microorganisms or absence of such microorganisms

In rat cecum contents the bile acids are present partly adsorbed and partly bound in a nondialyzable form to the intestinal residue (2,4) Microbial as well as dietary factors seem to be of importance for these processes (4)

Received 18 x.71

TABLE 1 *Chemical Names of Chenodeoxycholate Metabolites*

Acid	Abbrevation
3α, 7α-Dihydroxy-5β-cholanoic acid (chenodeoxycholic acid)	3α OH, 7α-OH
3 Keto-7α-hydroxy 5β-cholanoic acid	3-keto, 7α-OH
3β, 7α-Dihydroxy 5β-cholanoic acid	3β-OH, 7α-OH
3β Hydroxy-7 keto-5β-cholanoic acid	3β-OH, 7-keto
3, 7-Diketo-5β-cholanoic acid	3, 7-diketo
3α Hydroxy-7-keto-5β-cholanoic acid	3α-OH, 7-keto
3β, 7β-Dihydroxy-5β-cholanoic acid	3β-OH, 7β-OH
3-Keto-7β hydroxy-5β-cholanoic acid	3-keto, 7β-OH
3α, 7β-Dihydroxy-5β-cholanoic acid	3α-OH, 7β-OH
3α-Hydroxy 5β-cholanoic acid (lithocholic acid)	3α-OH
3-Keto-5β-cholanoic acid	3-keto
3β Hydroxy-5β-cholanoic acid	3β-OH

TABLE 2 *Chromatographic Systems*

Phase system	Moving phase	Millilitres
S 10	Trimethylpentane isopropyl alcohol acetic acid	60 20 0 5
S 12	Trimethylpentane ethyl acetate acetic acid	5 25 0 2
S 15	Trimethylpentane ethyl acetate acetic acid	20 20 0 2

The present experiments were designed in order to determine the distribution of labelled bile acids and their microbial formed metabolites between the supernatant and the sediment after centrifugation of broth cultures of rat intestinal microorganisms Additionally, various factors affecting the microbial 7α dehydroxylation as well as the adsorption of bile acids to the microorganisms were studied

MATERIALS AND METHODS

Bacteriological Procedures

Source of intestinal contents Three adult, male conventional rats were used Collection as well as dilution of intestinal contents were performed as described elsewhere (8 9)

Culture technique Unless otherwise stated the cultures were grown anaerobically in Todd Hewitt broth (Oxoid) (TH broth) at 37° C for 7 days Anaerobic conditions (an) were obtained using the pyrogallol method (3) In order to cultivate aerobic and aerotolerant bacteria, aliquots from all the dilutions steps were spred on series of blood agar plates (B-A) Mitis Salivarius agar plates (MS-A) and Endo agar plates (E A) The plates were incubated aerobically (a) at 37° C and inspected daily for growth up to 3 days

Strain The 7α-dehydroxylating strain used

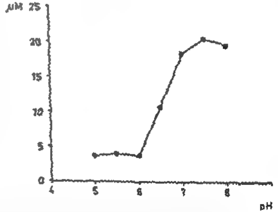

Fig 1 Maximum concentration of lithocholic acid/lithocholate in TH broth of different pH

Th broth, phosphate buffer and N NaOH or HCl was added to give the regular concentration of TH phosphate buffer ($^1/_{15}$ M) and the pH selected The broth was sterilized by filtration through a Millipore type GS filter and added to tubes conta...
The tu...
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the isotope content was determined in the supernatant and the maximum concentration of lithocholic acid/lithocholate at different pH was calculated

Strain II, has previously been isolated from rat feces (3), tentatively identified as an anaerobic lactobacillus (6), and its action on bile acids has been described (3, 7)

Chemical Procedures

The source and references to synthesis of 24-^{14}C labelled and unlabelled free and conjugated bile acids have been given previously (3, 7) The names and abbrevations of bile acids used are given in Table 1

Determination of microbial transformation products of chenodeoxycholic acid and lithocholic acid The cultures were centrifuged for 1 hour at 25,000 g and the supernatant decanted The sediment was washed with 0 5 ml of a $^1/_{15}$ M phosphate buffer with a pH the same as determined in the supernatant, and centrifuged as above The washed sediment was extracted by refluxing in acetone The supernatant and phosphate washings were combined, acidified with hydrochloric acid, and the labelled bile acids extracted with ether The residue remaining after the acetone extraction was dissolved in water, acidified, and the labelled bile acids extracted with ether The ether extracts were evaporated, dissolved in acetone, and aliquots taken for chromatographic analysis or isotope determination

The labelled metabolites were separated with thin layer chromatography (TLC) using the phase systems *S 10*, *S 12*, and *S 15* (Table 2) Radioactive spots were located by autoradiography For quantitation, the optical density of the film at 550 μu was measured (7, 10) Spots of unlabelled bile acids were visualized by spraying with concentrated sulphuric acid and charring at 250° C.

Determination of splitting of conjugated bile acids The broth cultures were incubated for 7 days with either 1mM of taurodeoxycholic acid or glycocholic acid The method for determination of unconjugated bile acids has been described previously (7)

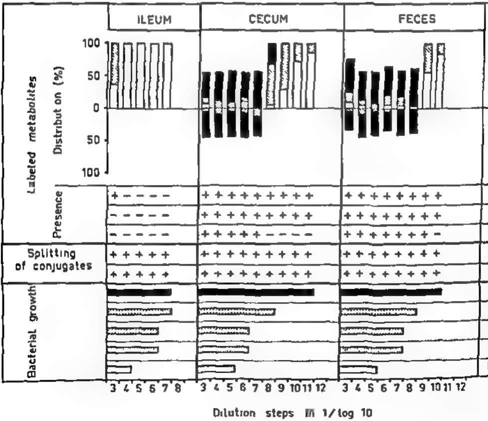

Fig 2 Rat No 1 Microbial bile acid transformation and bacterial growth in cultures of dilutions of rat intestinal content from ileum and cecum and from feces
Labelled bile acids ▭ unchanged chenodeoxycholate ▭ metabolites with hydroxyl or keto group at C-7, ■ metabolites without hydroxyl or keto group at C-7 All specimens were diluted 10^{12} The data given refer to dilution steps showing growth For abbreviations see Materials and Methods.

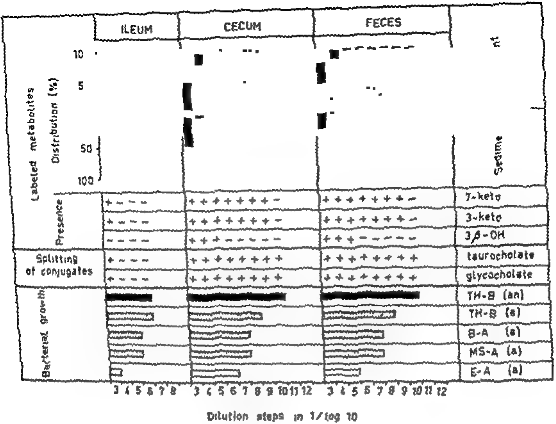

Fig 3 Rat No 2 For explanation see Fig 2

RESULTS

Solubility of Lithocholic Acid/Lithocholate in TH Broth of Different pH

The results (Fig 1) show that lithocholic acid is sparingly soluble in TH broth of pH below 6 0 In cultures of intestinal micro-organisms the pH was regularly found to be above 7 0 Due to the low solubility of lithocholate the original bile salt concentration in the following experiments was not above 10 μM

Distribution of Metabolites of Chenodeoxycholate in Broth Cultures of Intestinal Microorganisms

Microbial transformation of chenodeoxycholate-24-^{14}C and bacterial growth in dilutions of intestinal content from ileum, cecum and feces were studied in three rats and the results are summarized in Figs 2–4

Bacterial growth All specimen were diluted 10^{12} The data recorded in Figs 2–4 refer to tubes showing growth In cecum contents and feces the anaerobes outnumbered the aerobes by 10^{2} or more The differences were not so pronounced in ileum contents Aerobic plate count determinations of ileum contents (Figs 2–4, lower parts) showed 10^{7}–10^{8} of streptococci and lactobacilli in all three rats, and only few enterobacteria could be demonstrated In cecum contents plate counts gave 10^{6}–10^{7} of streptococci and lactobacilli compared to 10^{7}–10^{8} in feces The numbers of enterobacteria, mainly *E coli*, were 10^{5}–10^{6} both in cecum contents and feces

Distribution of labelled metabolites in supernatant and sediment fraction After centrifugation of the broth the isotope in the supernatant and the sediment fractions was determined and the results are summarized in upper parts of Figs 2–4 No isotope was pre-

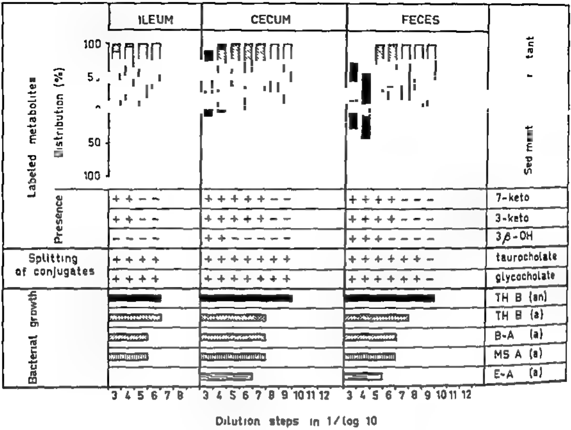

Fig 4 Rat No 4 For explanation see Fig 2

sent in the sediment of cultures of microorganisms from ileum contents In dilution steps 3 and 4 of cecum contents and feces labelled compounds in the sediment were regularly observed

Nature of metabolites formed The metabolites present in the supernatant and sediment were separated by TLC and quantified by densiometric recordings of the autoradiographs Representative chromatograms are shown in Fig 5 Labelled compounds were found at the places of [3β-OH, 7α-OH], [3α-OH, 7-keto], [3-keto, 7α-OH], [3α-OH], [3β-OH], and [3-keto] In the sediment fraction a band (A) of compounds more hydrophobic than [3-keto] was isolated These compounds consisted mainly of hydrosable derivates of 3β-OH The derivates were isolated by preparative TLC Weak hydrolysis (N NaOH) in 90 per cent ethanol (2 hours at 60° C) was found to decompose the derivates After hydrolysis, most of the labelled compounds had the chromatographic behaviour of [3β-OH]

Figs 2-4 give the percentage distribution of metabolities in sediment and supernatant In the sediment, the labelled compounds consisted mainly of metabolites without a 7α hydroxyl group, and only trace amounts of metabolites with a keto or hydroxyl group at C-7 were found [3α-OH], [3β-OH], and [3-keto] formed were also present in the supernatant

Physical State of 7α-dehydroxylated Metabolites of Chenodeoxycholate formed by Strain II in Cultures of Intestinal Microorganisms

Lithocholate formed by Strain II in anaerobic cultures has been shown to be present in the supernatant (3) In order to investigate

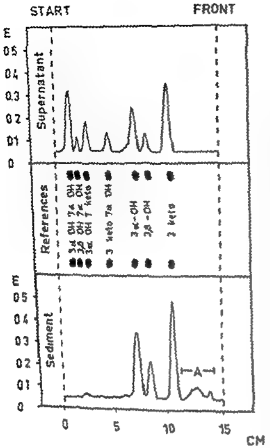

Fig 5 TLC separation of labelled metabolites in supernatant and sediment of a broth culture originally containing 10μM labelled chenodeoxycholate and incubated for seven days with dilution 10^{-3} from feces of Rat No 3 Densiometric recordings of the autoradiographs are shown in the upper and lower parts of the figure The reference substances were detected by spraying with sulphuric acid

the effect of mixed culture conditions, Strain H was added to all dilution steps The results from rat 1–3 were similar and only the results from rat No 2 are given (Fig 6) In all dilution steps a 7α dehydroxylation was obtained In addition to [3α–OH] and [3β–OH] [3 keto] was observed in all dilution steps Saponifiable derivatives of [3β–OH] were only seen in the lower dilution steps of cecum contents and feces In all dilution steps part of the labelled metabolites were recovered from the sediment, most pronounced in the lower dilution steps Thus intestinal contents contain high numbers of microorganisms to which [3α–OH], [3β–OH] and [3–keto] can be adsorbed

Effect of pH in Cultures on Microbial Action on Chenodeoxycholate and Lithocholate

Tubes containing either 10μM lithocholate-24-^{14}C or chenodeoxycholate-24-^{14}C in TH media of different pH were inoculated with intestinal microorganisms of dilution steps 10^{-3} from cecum content The distribution of labelled compounds between supernatant and sediment and the composition of metabolites were determined and the results from Rat No 1 are summarized in Fig 7

Types of metabolites formed The formation of metabolites in the cultures is given in the lower part of Fig 7 Removal of the 7α hydroxyl group did not occur in media with initial pH below 6 0 Formation of [3–keto] and [7–keto] derivatives was found in all cultures, but was more pronounced above pH 6 0

Distribution of metabolites between supernatant and sediment The sediment fraction always contained labelled compounds (Fig 7, upper part) In the experiments with lithocholate increasing amounts of labelled compounds were present in the sediments when pH was altered from 7 5 to 5 0 This was partly due to decreased solubility of lithocholic acid In the experiments with chenodeoxycholate-25-^{14}C, metabolites without hydroxyl or keto group at C-7 were found only in sediment fractions of cultures with pH ≧ 6 5 At pH below 6 0, the amount of labelled compounds in the sediment decreased due to the lack of 7α dehydroxylation in these cultures The sediment fractions of these cultures consisted mainly of unchanged chenodeoxycholate

DISCUSSION

Previously it was shown that deoxycholic acid and lithocholic acid formed in mixed cultures of intestinal microorganisms from cholic acid and chenodeoxycholic acid respectively were

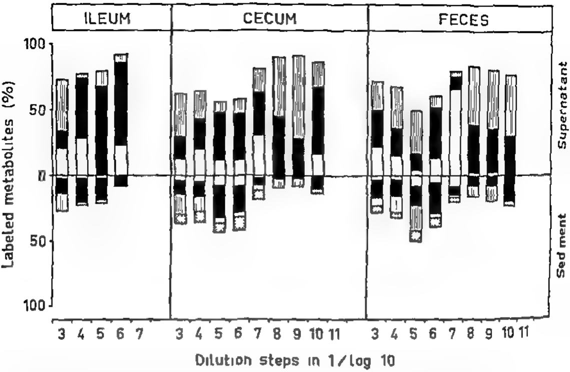

Fig 6 Microbial transformation of chenodeoxycholate in cultures of the dilutions shown in Fig 3 together with Strain II Labelled bile acids unchanged chenodeoxycholate and metabolites with hydroxyl or keto groups at C–7 [3α OH] and [3β OH] (3 keto) saponifiable derivatives of (3β–OH) Aliquots of 0 25 ml of the anaerobic broth cultures were inoculated with 0 25 ml of broth culture of Strain II into tubes containing 4 5 ml TH broth with 10μM chenodeoxycholic acid 24 ^{14}C

present in different physical state (3, 11) Centrifugation of the cultures showed that lithocholic acid was present mainly in the sediment whereas deoxycholic acid was present mainly in the supernatant The formation of lithocholic acid from chenodeoxycholic acid has been studied in pure cultures of an anaerobic lactobacillus (3 7) It was shown that all of the formed lithocholic acid was present in the supernatant fractions of the centrifuged cultures The difference in physical state of lithocholic acid in cultures of intestinal microorganisms together with a strain of anaerobic lactobacilli capable of 7α dehydroxylation has been studied in the present work The results indicate that lithocholic acid and its metabolites [3β–OH] and [3-keto] are adsorbed to microorganisms other than those which are responsible for its formation These microorganisms capable of adsorption of lithocholic acid were shown to be present in high numbers in rat intestinal contents No similar adsorption of chenodeoxycholic acid and its metabolites with keto groups at C–7 was demonstrated

It has been shown earlier that lithocholic acid was adsorbed to residue of intestinal contents from germfree rats (4) The present findings indicate that lithocholic acid is also adsorbed to intestinal microorganisms It is reasonable to assume that both mechanisms take place *in vivo*

Part of the investigation was done during a stay as visiting scientist at the Department of Germfree Research Karolinska Institutet Stockholm Sweden

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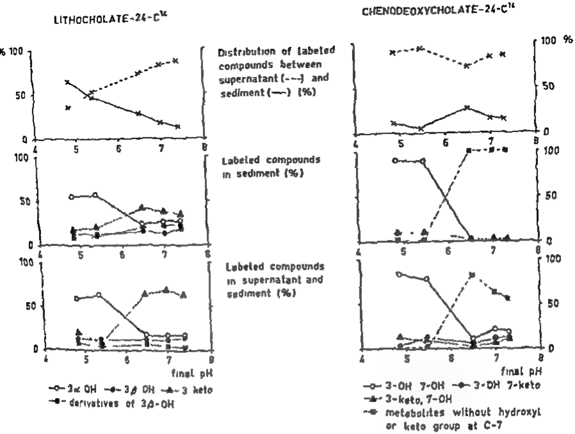

Fig 7 Effect of variations of the media pH in growing cultures upon the transformation of chenodeoxy-chol...

...dilution step 10^{-3} from the cecum content of Rat No 1, the cultures were incubated anaerobically at 37° C for seven days Bacterial growth was obtained in all tubes The final pH of the cultures was determined immediately after removal of the anaerobic seals

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ANTIGEN-FREE MEDIUM FOR CULTIVATION OF *HAEMOPHILUS INFLUENZAE*, AFH-MEDIUM

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An antigen free medium, AFH medium, for cultivation of *H influenzae* is described The medium is prepared from trypticase yeast autolysate, filtrated through a Sephadex G-25 column For the medium base only the fractions containing low molecular material were utilized Addition of different amounts of salt and buffer was tested and the effect of the initial pH of the medium was studied The amount of glucose consumed during growth was measured with a glucose oxidase test The AFH medium provided luxuriant growth of all tested strains The bacterial yield in the fluid medium was 1×10^{10}–1×10^{11} per ml and overnight cultures on the solid medium resulted in colonies 1–2 mm in diameter

Media commonly used for cultivation of *H influenzae* are based on a nutritient broth supplemented with 5–10 per cent defibrinated horse blood which contains the necessary growth factors haematin and nicotinamide-adenine dinucleotide (NAD) in addition to other growth supporting material Haematin and NAD are released from the blood corpuscles either by heat as in Levinthal medium and haematin agar medium or by digestion with trypsin (*Levinthal* 1918, *Fildes* 1921)

These complex media contain substances of high molecular weight which have antigenic properties Despite repeated washings of bacteria grown in such media it is often difficult to secure them totally free from the medium substances When such bacteria are used for production of antisera, antibodies may be formed against the contaminating medium substances as well and this may cause difficulties in the analyses of the bacterial antigen preparations An antigen-free synthetic medium would therefore be preferable for growing bacteria to be used for serological studies and have been developed for the cultivation of *H influenzae* (*Talmadge & Herriot* 1960, *Butler* 1962) The growth of *H influenzae* in these media is sparse and slow which limits the amount and possibly the nature of the antigens produced *Holm and Falsen* (1967) developed for β-haemolytic streptococci an antigen-free medium, trypticase-yast autolysate (Ty G-25) which contained only molecules less than 2500 (filtration through Sephadex G-25)

In preliminary tests with Ty G-25 + haematin no growth was obtained of some tested *H influenzae* strains However, when the medium was diluted with an equal amount of physiological saline some growth was obtained In order to investigate whether a better growth supporting capacity of a medium

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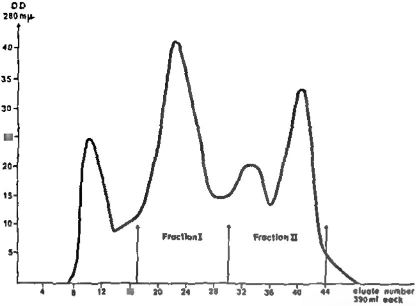

Fig 1 The UV absorption curve of the TYN solution filtrated on the Sephadex G 25 column (15 3 × 53 cm) Fraction I and Fraction II constituted the STYN solution used as base for the AFH medium

of this type could be obtained the following experiments were performed

MATERIAL AND METHODS

Bacterial strains

For the elaboration of the antigen free medium (AFH medium) the following Haemophilus influenzae strains were used

1 Strain Smith type a (designated Ma)
2 A nonencapsulated variant of strain Smith (designated Sa)
3 Strain Montenegro type e (designated Me)

For testing the efficacy of the AFH medium the following encapsulated strains were used strain RAB (type b) NCTC 8469 (type c), strain Garf (type d) NCTC 8455 and NCTC 8472 (type e) NCTC 8473 (type f) and in addition fresh isolates from haematin agar or blood agar plates of 100 encapsulated *H influenzae* strains representing the capsular types a b d e and f and 200 nonencapsulated H influenzae strains

Media

Trypticase used in the preparation of B D Merieux

Yeast autolysate prepared according to the description of *Holm and Falsen* (1967)

NAD solution *Alexander's* method (1) for extraction of NAD from baker's yeast was utilized and modified as follows Baker's yeast, 150 g were boiled for 15 minutes in 300 ml distilled water cooled clarified by centrifugation at 2000 × g for 15 min The supernatant was removed and stored at —25° C if not used immediately

Haematin histidine A stock solution of haematin (pure haematin Koch Light Lab) and histidine (Fluka) was prepared by dissolving 0 1 g haematin and 0 1 g histidine with the aid of 2 g triethanolamine (Merck) per 100 ml distilled water and heating the mixture to 60° C for 10 minutes (at the suggestion of H Leidy, Colombia University N Y 1966) The stock solution was sterilized by membrane filtration and stored at —25° C until used One ml of this solution was added per 100 ml of sterile medium just before inoculation of the cultures

$NaHCO_3$ A 4 per cent solution of $NaHCO_3$ was prepared and sterilized by membrane filtration To 100 ml medium 0 5 ml of the solution was added when the tests were performed A new $NaHCO_3$ solution was prepared every second week

TYN solution Yeast autolysate, 1600 ml and NAD solution 300 ml were combined and concentrated to 1000 ml in a Buchi Rotavapor at 33° C If the solution was not quite clear after the concentration it was centrifuged at 2000 × g for 20 min Thereafter 125 g trypticase (B D Merieux) were added and dissolved by gentle warming to about 40° C

STYN solution (Sephadex filtrated TYN solution) Gelfiltration was performed at 4° C on a 15 3 × 53 cm column packed with fine bead polymerized Sephadex G 25 (Pharmacia, Sweden) One litre of TYN solution was placed on the column and eluted with 0 02 M potassium phosphate buffer pH 7 4 (3 4 g/l) at a flow rate of 310 ml per hour The effluent was collected in 390 ml portions with an automatic collector The optical density of each fraction was measured at 280 mμ in a Beckman

DB spectrophotometer The material in the first absorption peak (the high molecular weight material) was discarded The remaining material (the STYN solution) was for some experiments divided into two equal parts designated Fraction I and Fraction II (Fig 1)

The STYN solution or various combinations of Fraction I and II with different combinations of the variable additives listed below were sterilized by filtration (Sartorius Membranfilter 0 1 μ) after a final adjustment to pH 7 4 with NaOH The sterilized solutions were stored in 100–200 ml portions at —25° C until used

Variable additives to the STYN solution Sodium chloride was added in amounts of 0 3 g 0 5 g or 0 7 g per 100 ml STYN solution. Glucose was added in amounts of 0 2 g 0 4 g 0 6 g or 0 8 g per 100 ml STYN solution Potassium phosphate was added in amounts of 0 2 g (KH_2PO_4 0 04 g + K_2HPO_4 0 16 g) 0 3 g (KH_2PO_4 0 06 g + K_2HPO_4 0 24 g) or 0 4 g (KH_2PO_4 0 08 g + K_2HPO_4 0 32 g) per 100 ml to prepare solutions of pH 7 4

Fluid PC medium was prepared as follows

A.	STYN solution (Fraction I and II 1 1)	1000 ml
B	NaCl	7 g
	Glucose	4 g
	KH_2PO_4	0 6 g
	K_2PHO_4	2 4 g
C	Haematin histidine 0 1 per cent solution)	10 ml
	$NaHCO_3$ (4 per cent stock solution	5 ml

B was added to A and the pH adjusted to 7 4 with NaOH The medium base was then sterilized by membrane filtration and stored at —25° C The supplements under C were added from sterile stock solutions just before the medium was to be used

Solid PC medium was prepared by adding sterile fluid PC medium to an equal amount of 3 per cent Bacto agar autoclaved in fluid PC medium and pouring the mixture into Petri dishes

Fluid AFH medium was prepared as fluid PC medium except that 6 g glucose was added instead of 4 g

Solid AFH medium was prepared from equal parts of sterile AFH medium and 3 per cent Bacto agar autoclaved in AFH medium

Levinthal medium Fluid and solid (1 5 per cent agar) Levinthal media used as control media were prepared according to a modification of *Alexander* (1)

Cultivation Procedures

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aga plate to fluid PC medium and incubated for 10 hours at 35° C The medium to be tested 25 50 ml in a 200 ml Erlenmeyer flask was inoculated with 0 25 0 5 ml (1 per cent of volume) *of the preculture* All fluid cultures were incubated at 35° C and aerated with a Gyrotory shaker (New Brunswick Scientific Company, N J) Samples were removed from the cultures intermittently and the turbidity pH and viable cell counts were measured The turbidity (OD) of the culture was measured in a Beckman C colorimeter at 660 $m\mu$ The cultures were appropriately diluted when the turbidity exceeded an extinction of 0 7 By means of a pH meter (Philips PR 9400) the pH of the cultures was measured

Viable cell counts (VC) were performed by making two tenfold dilution series of each culture in the fluid PC medium From each of the dilutions to be tested (usually three in each series) 0 2 or 0 4 ml were placed in each of two Petri dishes and mixed with about 20 ml of melted solid PC medium The number of colonies on each plate was counted between the 24th and 36th hour of incubation at 35° C The counts are expressed as the average number of colonies on at least two plates

The amount of glucose in the cultures was determined in duplicate with glucose oxidase (Glox, Kabi Sweden) according to a method by Keston 1956 (5) developed for determination of glucose in blood

Cultures were prepared from solid Levinthal medium and transferred to fluid Levinthal medium as was done for the PC medium The cultivation procedure and the different tests were made in a similar manner as for the PC medium

RESULTS

In the initial experiments addition of haematin histidine, dilution with physiological saline and adjustment of the Ty G 25 medium of *Holm and Falsen* to pH 7 4 supported growth of *H influenzae* to an OD of about 1 2 With these adjustments the amount of NAD in the Ty G 25 medium was growth limiting and addition of pure NAD increased the OD to about 1 6 For this reason a crude NAD solution was added to the trypticase-yeast autolysate solution used by *Holm and Falsen* The amount of NAD added seemed to be sufficient since in no test with the PC medium did addition of pure NAD result in a greater yield of bacteria

In preliminary tests the STYN solution

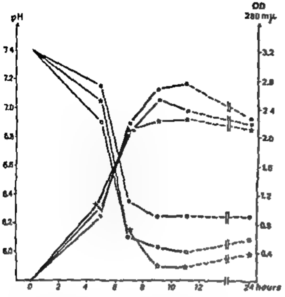


Fig 2 Effect of addition of different amounts of potassium phosphate on the OD and pH values in an experiment with strain Sa
0 2 g/100 ml OD ★—★, pH ☆—☆
0 3 g/100 ml OD ■—■ pH □—□
0 4 g/100 ml OD ●—● pH ○—○

provided acceptable growth and was therefore used as a precultivation medium (PC medium) in subsequent studies

Different amounts of haematin were not

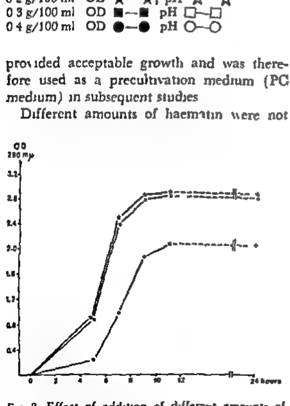


Fig 3 Effect of addition of different amounts of NaCl on the OD values in an experiment with strain Me
0 3 g/100 ml ●—●
0 5 g/100 ml ■—■
0 7 g/100 ml ★—★

tested but the amount used was comparable to that used in other *H influenzae* media

Experiments with Fluid PC Medium

Effect of potassium phosphate concentration The effect of addition of different amounts of potassium phosphate, 0 2 g 0 3 g and 0 4 g per 100 ml fluid PC medium was tested with the Ma, Sa and Me strains All three strains gave similar results Fig 2 shows the OD and pH values over a 24 hour period with strain Sa The results indicate that the highest OD (at 9–10 hours) was obtained with the addition of 0 4 g phosphate The drop in pH was least with this amount of phosphate The cultures with 0 2 g phosphate had the lowest OD and pH ratings, while the values for 0 3 ranged between those for 0 2 g and 0 4 g Repeated experiments showed, however, that the cultures with 0 4 g had a longer lag phase than those with 0 2 or 0 3 g phosphate Occasionally the cultures did not grow in the medium containing 0 4 g phosphate

Effect of sodium chloride concentration The effect of sodium chloride, 0 3 g, 0 5 g and 0 7 g per 100 ml in the fluid PC medium was tested The results which were very similar for the three strains revealed that, upon the addition of 0 3 g NaCl, the OD was lower than and sometimes only half of that obtained when 0 5 g or 0 7 g were used The latter two gave similar results, as illustrated in Fig 3 with strain Me When half the amount of 0 7 g NaCl was replaced with KCl the OD was lower reaching the same level as when 0 3 g NaCl was added

Effect of glucose concentration In a series of experiments with strains Ma Sa and Me using the fluid PC medium the consumption of glucose was estimated by means of the glucose oxidase test The results showed that all glucose was consumed within 5–7 hours In other experiments using strain Ma the effect of addition of 0 2 g 0 4 g 0 6 g and 0 8 g glucose per 100 ml medium was tested In these experiments OD, VC pH and consumption of glucose were followed over a period of 24 hours growth With addition of

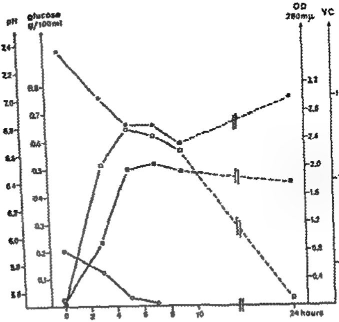


Fig 4 a

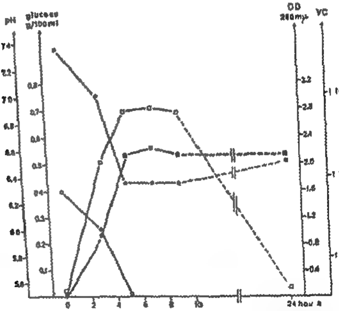


Fig 4 b

0 2 g glucose growth stopped when all glucose had been consumed and the whole amount was consumed within 4–5 hours The greater quantities of glucose (0 4 g, 0 6 g or 0 8 g) allowed a development of optical densities and viable cell counts higher than those to develope with 0 2 g glucose Slightly higher optical densities and viable cell counts values resulted when 0 6 g or 0 8 g glucose rather than 0 4 g was used The addition of 0 8 g glucose which was not totally consumed caused a much greater drop in pH (to about 5 5–5 6) than with 0 4 g or 0 6 g glucose (to about 6 0–6 4) The viable cell counts also showed that the death rate of the cultures was much faster with 0 8 g glucose

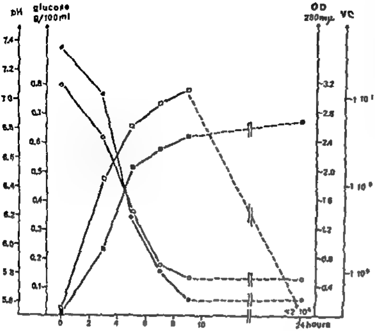


Fig.s 4 Effect of addition of different amounts of glucose on the OD, VC and pH values in an experiment with strain Ma In addition the glucose consumption during growth is registered Initial amounts of glucose 4a (0 2 g) 4b (0 4 g), 4c (0 8 g) glucose O—O, OD ■—■, VC □—□ pH ●—●

Fig 4 c

than with the lower amounts Fig 4a, 4b and 4c illustrate an experiment with strain Ma

Effect of different proportions of Fraction I and II A series of experiments was performed with strain Ma, Sa and Me using different combinations of Fraction I and II with 0 4 g glucose, 0 7 g NaCl and 0 3 g phosphate per *100 ml* medium

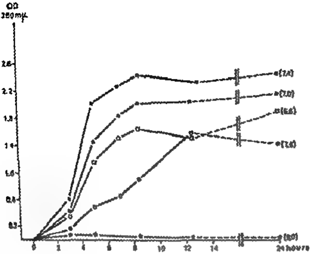
Table 1 presents the results of a typical experiment where the OD was recorded after 9 hours of growth which corresponded to the end of the log phase Variation of the proportions of the Fraction I to Fraction II had little effect on the growth of the three test strains

Effect of different initial pH A series of experiments with strains Ma, Sa and Me using the PC medium adjusted to different pH, ranging from 6 6–8 0 was performed The results showed that the highest OD were obtained with PC medium pH 7 4 With an initial pH of 7 8 or 8 0 no growth was obtained and with pH 7 6 the OD was considerably lower than with pH 7 4 Also with initial pH less than 7 4 the OD were lower Fig 5 illustrates an experiment with strain Ma

AFH medium

When the growth supporting effects of the fluid AFH medium was tested, repeated experiments with a single batch of the medium showed very good reproducibility Different batches of media, however, differed slightly in their growth supporting capacity Table 2 shows the range and the average OD measured after 9 hours cultivation of the three test strains in 37 experiments performed over a period of 2 years The results with Levinthal medium represent early experiments when this medium was used as control It is apparent from Table 2 that all three tests strains attained higher optical density in the AFH medium than in the Levinthal medium

The *H influenzae* type strains listed in Table 3 were cultivated in mass cultures for the production of bacterial antigen Four different batches of AFH medium were used (with addition of 4 g instead of 6 g glucose per litre of medium) Five to ten 2 l Erlenmeyer flasks, each containing 500 ml of medium were inoculated for each strain The cultures were grown as before on the Gyrotory shaker for 9–10 hours The flasks of a single



Figs 5 Effect of initial pH of the medium on the OD and pH values in an experiment with strain Ma Fig 5a OD, Fig 5b pH pH 6 6 □—□, pH 7.0 ●—●, pH 7 4 ■—■, pH 7 6 ○—○, pH 8 0 ★—★

Fig 5 a

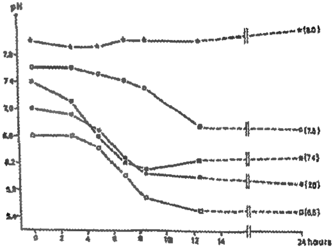


Fig 5 b

strain were pooled centrifuged at 10 000×g for 30–40 min (the cultures of the heavily encapsulated Ma strain were centrifuged at 25 000×g for 40 min) and the bacterial sediment harvested The OD and pH of each culture were determined prior to centrifugation and the wet weight of the cells measured It can be seen from Table 3 that the OD and the wet weight of the bacteria were not necessarily correlated For example similar OD values were obtained for the heavily encapsulated Ma cultures and the nonencapsulated Sa variant while the weight of the Ma strain sediment was nearly twice that of the Sa strain

All newly isolated patient strains both encapsulated and nonencapsulated grow well in the fluid AFH medium The bacterial yield for these strains compared favourably with that of the type strains

Solid PC and AFH Media

The PC and AFH media solidified with 1 5 per cent agar provided at least as good

TABLE 1 *Optical Densities of [illegible] Hour Cultures Using Different Combinations of Fractions I and II*

Strain	Fraction I II 5 1	3 1	2 1	1 1
Ma	2 56	2 72	2 80	2 64
Sa	2 88	2 88	2 80	2 72
Me	2 16	2 48	2 56	2 53

TABLE 2 *Optical Densities of 9 Hour Cultures with AFH Medium and with Levinthal Medium*

Strain	AFH medium			Levinthal medium		
	No of tests	OD		No of tests	OD	
		mean	range		mean	range
Ma	14	2 2	1 8–2 6	4	1 7	1 6 1 8
Sa	11	2 2	1 8–2 8	4	1 65	1 6 1 7
M•	12	2 35	1 9 2 9	5	1 7	1 5 1 8

TABLE 3 *Optical Density, Final pH and the Yield of Bacteria of Type Strains Cultivated in Amounts of 2 5 5 0 Litres for Antigen Production*

Strain	Batch	OD		Final pH	Wet sediment gram/litre
		mean	range		
Ma (Smith)	I	1 8	1 5–2 0	6 0	25
Sa (Smith)	I + II	1 9	1 5–2 0	5 8	14
Mb (RAB)	II + III	2 0	1 65–2 1	6 0	12
Mc (NCTC 8469)	III	2 2	2 05–2 4	5 9	13
Md (Garf)	II	1 9	1 55–2 05	5 8	11
Me (NCTC 8455)	IV	1 7	---	6 7	20
Me (NCTC 8472)	IV	2 4	2 1 2 65	6 7	[illegible]
Mf (NCTC 8473)	III	2 4	–	6 2	22

growth of the different *H influenzae* strains as the Levinthal agar medium Colonies of the encapsulated strains had a diameter of about 2 mm after 20–24 hours of growth were opaque showed strong iridescense and were somewhat more mucoid and confluent than on Levinthal agar The colonies of non encapsulated strains were generally 1–1 5 mm in diameter transluscent exhibiting a smooth surface and sometimes expressing a tendency to be confluent The tendency to confluence was especially evident on the AFH medium containing the higher amount of glucose

DISCUSSION

The proper choice of growth medium is a prerequisite for all serological work with bacteria The primary demand is that the growth of the bacteria should be luxuriant enough to provide a satisfactory yield of antigenic material Secondly it is desirable that the medium be nonantigenic to facilitate the immunologic analyses For the latter reason synthetic media composed of low molecular weight well defined, growth promoting substances would be preferred However for fastidious bacteria such as *H influenzae* the growth in synthetic media described (*Talmadge & Herriot* 1960 *Butler* 1962) is poor

Media containing only low molecular but undefined substances may be obtained by dialysis of ordinary media A more convenient method for elimination of high molecular substances is filtration through Sephadex G 25 gel as used for the antigen free medium of Holm and Falsen employed for the cultivation of *β haemolytic streptococci* The choice of a trypticase yeast autolysate base for their medium was well founded since many growth promoting nutritient substances including vitamins and minerals are present in the yeast cells However the streptococcal medium of Holm and Falsen with addition of haematin and adjustment of pH to 7 4 did not promote any growth of *H influenzae* bacteria When the medium which contained a very high amount of $NaHCO_3$ but no NaCl was diluted with an equal amount of physiological NaCl a moderate growth was obtained Th■

growth could be somewhat further increased by addition of pure NAD

Thus various factors seemed to limit the growth supporting capacity of the streptococcal medium for *H influenzae* bacteria For the development of an antigen free *H influenzae* medium it was therefore decided to use the trypticase yeast autolysate of Holm and Falsen as medium base By variation in the addition of some of the supplements *e g* NAD glucose salt and buffer the effect on the bacterial yield was studied

Since the amount of NAD in the streptococcal medium was found to be a growth limiting factor a yeast extract solution from bakers yeast was added as an inexpensive source of NAD It was added to the trypticase yeast autolysate solution before the Sephadex filtration in order to exclude from the NAD solution all high molecular material The NAD added in this way seemed to be a sufficient amount since addition of pure NAD did not increase the growth in any test with the PC or the AFH medium

The low molecular material eluted from the Sephadex column was for some experiments collected in two equal parts Fractions I and II for testing the growth promoting effect of each fraction Fraction I contained according to *Holm and Falsen* (4) most of the amino acids and other growth promoting substances and they therefore used the whole amount of Fraction I but only ¼ of Fraction II for the streptococcal medium However for the AFH medium all the UV absorbing low molecular material (Fraction I and Fraction II) could be utilized with a satisfactory bacterial yield

The buffering capacity of the potassium phosphate used in the AFH medium (0 64 per cent) seemed to be somewhat low but was dictated by the longer lag phase of the cultures or even unability to grow when 0 74 per cent potassiumphosphate was added The initial pH of the AFH medium was found to have a great influence on the amount of growth and a pH of 7 4 gave the best results A slightly higher pH (7 6) considerably lowered the OD and prolonged the lag phase Also a lower initial pH than 7 4 resulted in lower OD of the cultures probably by too low buffering capacity of the medium

In contrast to the β haemolytic streptococci, *H influenzae* required NaCl as additive to the medium Though the function of NaCl was most likely to provide an adequate external osmotic pressure it is possible that both Na^+ and Cl are needed to some extent

The ability of *H influenzae* to utilize glucose as an energy source has been a matter of discussion among some authors (8) and it is generally believed that these bacteria utilize glucose slowly and only to a limited degree For this reason and to establish the optimal concentration of glucose in the medium experiments were performed where the consumption of glucose during growth was measured by means of glucose oxidase test (5) At an initial concentration of 2 g or 4 g glucose per litre the entire amount was consumed and the bacterial growth then stopped (aereated cultures) When 6 or 8 g glucose per litre was used, somewhat higher OD and VC values accompanied with lower pH of the cultures was obtained In cultures with 8 g glucose growth stopped before all the glucose was consumed probably because of the low pH value The viable counts at 24 hours showed that the cell death in a culture medium containing 8 g glucose was considerably greater than in a medium with lower amounts of glucose

The presented antigen free medium for *H influenzae* the AFH medium provided luxuriant growth of all tested strains With an 1 per cent inoculum a bacterial yield of 1×10^{9} 1×10^{10} viable bacteria per ml was recorded in 8–9 hours old cultures It is probable that addition of 8–10 g glucose per litre medium together with continous correction of the pH (with potassium phosphate)

This investigation was supported by a grant from the *Faculty of Medicine University of Goteborg*

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ORGAN TRANSPLANTATION IN THE RAT

The Importance of the Ag B (or H-1) Locus

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Kidney heart and skin-grafting were performed from individual donor rats belonging to segregating F2 hybrid populations to recipients of one of the parental strains in two combinations (AS × BN) F2 → AS and (Fischer × BN) F2 → Fischer Before grafting all donors were tested for presence or absence of the Ag B allele from BN by serotyping and by mixed lymphocyte culture reaction According to the one locus hypothesis one would expect that organs from F2 donors which carried the BN allele would be rejected acutely whereas organs from negative F2 donors would survive for a long time concurrently with acute or subacute rejection of skin grafts In both combinations acute or subacute rejection of the skin grafts occurred, whether or not the donor carried the foreign Ag B allele from BN and acute or subacute rejection occurred of all kidneys and hearts from BN positive donors except for a single kidney which survived for 197 days A very distinctly prolonged survival for both kidney and heart grafts was noticed in both combinations when the donor lacked the Ag B allele from BN However one exception occurred viz a grafted heart which was rejected after 62 days But in general the prediction came true and it may be concluded that rejection of kidney and heart grafts is the common rule when incompatibility at the Ag B locus exists while compatibility at this locus leads to permanent acceptance of organ grafts independent of incompatibilities at the weaker loci

The fact that the HL A locus is of overriding importance for the outcome of kidney transplantation in man is now universally accepted although the immunosuppressive treatment used in the clinical routine makes it extremely difficult to assess the role which minor histocompatibility loci might otherwise play

Fortunately inbred strains of rats offer a model for organ transplantation which permits a rigorous analysis of the role played by the major histocompatibility locus of this species the Ag B (or H 1) locus relative to the role played by minor loci

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The present data represent an application to this problem in kidney and heart transplantation of an experimental design which is basically similar to that employed in much earlier investigations of the number of loci involved in skin graft rejection in mice (*Barnes & Krohn* (2)) and rats (*Billingham & Silvers* (5)) *i e* the grafting between segregating F2 hybrids and parental strain animals In order to increase the precision of the analysis each F2 donor rat was serotyped prior to grafting in respect of its Ag B genotype Likewise its mitomycin treated blood lymphocytes were tested in mixed lymphocyte culture MLC) for their ability to stimulate the lymphocytes of the recipient strain According to our working hypothesis only foreign Ag B antigens would lead to

organ graft rejection while skin grafts would also be *rejected due to minor incompatibilities*

The results were very largely confirmatory

MATERIAL AND METHODS

Animals

Young adult inbred male rats weighing 200–350 grammes were used Recipients were of the AS or Fischer (F) strains, and donors were (AS × BN)F2 and (F × BN)F2 hybrids respectively Kidney, heart and skin was transplanted from individual donors to different recipients of the same inbred parental strain

The Fischer and BN rats were obtained from Dr *Sjögren*, Lund, Sweden, and the AS rats from Dr *N W Nisbet*, The Charles Salt Research Centre, The Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, England, and maintained by brother-sister mating in this laboratory

Serotyping

Haemagglutinating antisera specific for the respective Ag-B antigens of the parental strains were used for typing of the F2 hybrids The antisera were obtained by reciprocal immunization in the AS BN and F-BN strain combinations by means of skin grafting followed by i p injections of liver and spleen cells at weekly intervals

AS-anti-BN and BN anti AS sera were used for typing in the dilution of 1 2, whereas F-anti BN and BN-anti-F sera were used in the dilution of 1 100 in PBS

The erythrocyte suspensions were approx 2 per cent made up in Intradex (Glaxo) Two drops of antiserum diluted in PBS and two drops of the erythrocyte suspension were incubated at 37° C for two hours in small plastic tubes before macroscopical reading The same technique was used when sera from recipients of organ grafts were analysed for haemagglutinating antibodies The MLC tests were performed by Dr S Freiesleben Sørensen as described elsewhere (18)

Surgical Techniques

Microvascular suture techniques for kidney and heart transplantation (11,12,13) were used

Under anaesthesia with "Avertin" (CBr, CH OH with amylenhydrate, 24 mg/100 g body weight in 0 9 per cent saline The Bayer Products Co , Surrey England) the donor s right kidney and ureter were first removed, including the renal vessels with small cuffs of the aorta and the vena cava and with a small segment of the bladder Thereafter the heart was removed by ligating the superior and inferior caval veins the pulmonary veins and by cutting the aorta and the pulmonary artery without ligature Both the kidney and the heart were kept in saline at 4°–8° C *Prior to the* removal of the organs the donor received 250 units of Heparin in 1–2 ml saline i v

The contralateral kidney of the donor was removed for histological control

Not until then the kidney recipient was prepared by right sided nephrectomy, and the donor kidney was grafted by end to side anastomoses of the aortae and venae cavae below the origin of the left renal vessels The donor ureter was passed under the sigmoid colon through the mesenterium and was implanted in the recipient bladder with only a tiny patch of donor bladder attached

Then the heart was grafted to another recipient by connecting the donor aorta and pulmonary artery end-to side with the host's abdominal aorta and vena cava, respectively

The aorta anaestomosis was performed by using a 9–0 nylon suture, the vena cava anastomosis and the bladder anastomosis by using a 8 0 silk suture (Ethicon Inc , Somerville, New Jersey, U S A)

The total ischaemic times were rather long, for the kidney about 80 minutes, and for the hearts about 3 hours This was because of the fact that both the kidney and the heart were removed from the donors before the actual grafting of either was commenced

On day 6 after the operation a biopsy for histological examination was taken from the grafted kidney and, at the same time, contralateral nephrectomy was performed

The function of the grafted kidney was followed by serum creatinine determinations Rejected grafts were not removed for histological examination before death, while many of the long term survivors were killed for the sake of more reliable histology

The function of the grafted heart was followed by palpation through the abdominal wall and the time of rejection was registered as the day when the heart stopped beating It was then removed for histological examination

Skin grafting was performed by transplanting full thickness squares of approx 15 × 15 mm of abdominal skin to the dorsal thoracic wall of the recipient, and the grafts were registered as rejected when they appeared as scabs

Histological preparations Biopsies and removed organs were fixed in 4 per cent buffered formalin, and sections of paraffin-embedded material were stained with haematoxylin eosin

Controls Left-sided nephrectomy was performed in two control groups of 10 AS rats and 10 Fischer rats and the right kidneys removed for histological control 12 months later Sera were sampled for creatinine determinations

RESULTS

The survival data for the three kinds of grafts are given in Table 1 for the strain combination of AS BN and in Table 2 for the F-BN combination. A total of 102 organ transplantations were performed, of which 15 kidneys and 5 hearts were technical failures. But from all 51 donors at least one of these organs was grafted successfully.

It is obvious from the tables that, in both strain combinations acute or subacute rejection of the skin grafts occurred, whether or not the donor carried the foreign Ag B allele from BN.

TABLE 1 *Kidney, Heart and Skin transplantation from (AS × BN)F2 → AS*

Group	Presence of the Ag B allele		Survival times in days		
	from BN	from AS	Kidney	Heart	Skin
I	—	+	471*	–	14-30
	—	+	84§	191§	14-30
	—	+	458*	244	14-30
	—	+	–	464*	14 30
	—	+	–	462*	14-30
	—	+	10§	338*	22
	—	+	337*	337*	17
	—	+	–	335*	17
	—	+	330*	330*	17
	—	+	329*	329*	17
	—	+	303*		19
	—	+	308*	62	19
	—	+		307*	21
	—	+	300*		15
II	+	+	–	37	15
	+	+	10	36	15
	+	+	13	39	16
	+	+	6§	10	13
	+	+	12	28	16
	+	+	21	34	20
	+	+	12	31	17
	+	+		8	17
	+	+		24	17
	+	+		36	14
III	+		17	9	18
	+	—	197	36	17
	+	—		19	17
	+		10	16	19
	+	—	46	9	13

* Killed while the graft was functioning
§ Death from non-immunological reasons
Technical failures

TABLE 2 *Kidney, Heart and Skin transplantation from (Fischer × BN)F2 → Fischer*

Group	Presence of the Ag B allele		Survival times in days		
	from BN	from Fischer	Kidney	Heart	Skin
I	—	+	58§	285*	16
	—	+	–	284*	18
	—	+	91§	258§	14
	—	+	–	277*	14
	—	+	273*	249§	16
	—	+	–	85§	15
	—	+	231§	90§	15
	—	+	175§	252*	15
	—	+	–	137§	15
	—	+	183§	–	16
	—	+	230*	230*	15
II	+	+	11	12	10
	+	+	12	12	11
	+	+	8	14	12
	+	+	–	12	10
	+	+	24	14	10
	+	+	9	15	9
	+	+	11	–	12
	+	+	–	17	9
	+	+	12	16	12
	+	+	12	8	11
	+	+	26	12	10

* Killed while the graft was functioning
§ Death from non immunological reasons
– Technical failures

Group II in both tables demonstrates the fact that acute or subacute rejection occurred of all kidneys and hearts from BN positive heterozygous donors. The same is seen, with one exception in Group III of Table 1, where the donors were BN positive homozygotes. This exception shows kidney survival for 197 days at which time the recipient died from a severe hydronephrosis without significant signs of rejection. This result contrasts with the fact that the heart from the same donor stopped beating after 36 days and showed on histological examination all characteristic signs of rejection.

On the average the hearts in Group II in the AS BN combination (Table 1) were rejected more slowly than the kidneys, but no

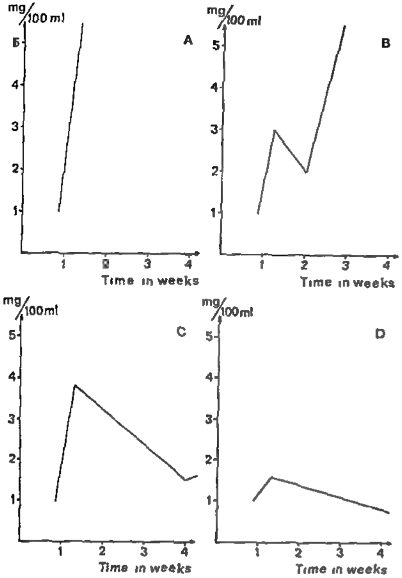


Fig 1 Summary of serum creatinine levels for the pooled kidney grafts in Tables 1 and 2 up to 4 weeks after transplantation *A* BN positive kidneys rejected acutely *B and C* BN positive kidneys rejected more slowly *D* BN negative kidneys not rejected

similar difference was seen in the F-BN combination (Table 2)

The faster rejection of hearts from BN-positive donors in the F BN combination than in the AS-BN combination is parallelled by the results of the skin grafting

Group I shows in both strain combinations very distinctly prolonged survival for both kidney and heart grafts, as expected since the donors lacked the Ag-B allele from BN Most of the recipients were killed while the grafts were intact and functioning without signs of rejection However there is one certain exception in Table 1 where the function of the grafted heart ceased after 62 days with very clear histological signs of rejection with mononuclear cell infiltration and muscular necrosis The kidney from the same donor showed perfect function up to day 308 when the recipient was sacrificed There is a more doubtful exception in the same group, where the heart stopped spontaneously after 244 days but showed a nearly normal histological picture

Some of the recipients in Group I in both strain combinations but especially in the F BN combination died at a relatively early stage but in all of these cases death was

deemed to be caused by non immunological complications, notably by pneumonia Fatal hydronephrosis only occured in 2 cases

Renal function as manifested by serum creatinine levels is summarized in Fig 1, for the pooled kidney grafts of Tables 1 & 2. It should be noted that all curves show a rise in the period between one and two weeks after the transplantation (contralateral nephrectomy was as mentioned before performed at day 6 after the transplantation) However, the rise in curve D is much lower than in curves A, B and C and similar to the rise seen in isografted recipients Therefore, it is probably caused by the operative trauma (including the ischaemic period)

As for the recipients rejecting the kidney more slowly (B & C) there is a higher initial rise in the serum creatinine level followed by a decrease, and a secondary increase prior to the death of the animal

The serum creatinine levels at the time of sacrifice of the 10 kidney grafted long-term survivors in groups 1 from Tables 1 & 2 which were killed while their grafts were *still* functioning is shown in Fig 2 A It shows normal levels in all but one case where there is a slight increase to 1 8 mg per cent Fig 2 B shows, by way of comparison, the values found in a group of 8 AS rats which were killed 12 months after unilateral nephrectomy as the only operation They actually show higher values than the graft recipients

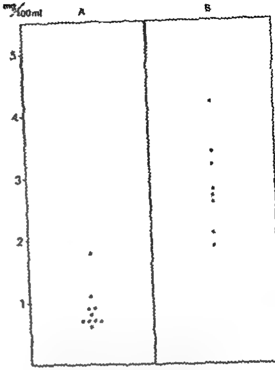


Fig 2 Serum creatinine levels at the time of sacrifice of *A* 10 kidney grafted long term survivors killed 8–15 months after grafting *B* 8 control animals killed 12 months after a unilateral nephrectomy was performed

Histology

All contralateral kidneys from the donors had a normal appearance, which indicates that in all cases we probably transplanted a *normal* kidney Nevertheless, some of the long-term surviving kidneys in the AS-BN *combination* showed, when removed about a year after grafting, signs of a slight cronic nephritis with areas of dilated tubules lined by flattened epithelium and filled with dense eosinophilic colloid material (Fig 3) However, this was *also* seen, to a much higher degree in the remaining kidney of all the unilaterally *nephrectomized* AS controls (Fig 4) but in none of the Fischer controls It seems as if the AS rats have a predisposition to chronic nephritis, probably caused by proliferation of cells in different parts of the nephrons with ensuing obstruction of the tubules (8) This also explains the above-mentioned high serum creatinine levels in the AS control group (Fig 2 B)

Table 3 summarizes the main body of histological findings

Regarding the organs from BN-positive donors we notice the classical signs of rejection with mononuclear cell infiltration and moderate tubular necrosis in the kidney biopsies taken at day 6 after the transplantation (Fig 5) In the kidney autopsies sligh to moderate mononuclear cell infiltration i seen in the AS-BN combination, and moderate to severe mononuclear cell infiltratior in the F BN combination In both combinations there is necrosis of the tubules anc obliteration of the glomeruli with eosinophili

TABLE 3 *Summary of Histology of Kidneys and Hearts Grafted from (AS × BN)F2 → A (Fischer × BN)F2 → Fischer*

Donor	Recip	Kidney biopsy		Kidney autopsy		Heart au
		cellular infiltr	necrosis	cellular infiltr	necrosis	cellular infiltr
BN neg	AS	– → (+)	–	– → (+)	–	– → +
BN neg	Fischer	– → (+)	–	– → +	–	–
BN pos	AS	+ → + +	+	– → +	+ + +	+ + +
BN pos	Fischer	+ + → + + +	+ +	+ + → + + +	+ + +	+ + +

(+) Very slight
+ *Slight*
+ + Moderate
+ + + Severe
* *One exception, see text*

material, (Fig 7), in the most severe cases complete infarction of the cortex

The terminal changes in the hearts grafted from BN positive donors are characterized by a severe mononuclear cell infiltration and a severe necrosis of the heart musculature with interstitial oedema or fibrosis (Fig 9) according as the rejection was acute or subacute

In great contrast to these findings stand the histological changes in organs transplanted from BN negative donors, where we only see a very slight perivascular mononuclear cell infiltration in some of the kidney biopsies (Fig 6) and, with one exception, no cell infiltration or necrosis in either kidney (Fig 8) or heart (Fig 10) at post mortem The exceptional heart from a (AS × BN)F2 donor which stopped beating at day 62 showed a heavy mononuclear cell infiltration and a severe muscular necrosis with interstitial fibrosis, in other words the classical signs of rejection More doubtful signs of cronic rejection were seen in the heart graft of the same combination which stopped beating after 244 days, and where a slight mononuclear cell infiltration and a slight interstitial fibrosis without any certain necrosis occurred

Antibodies

All sera tested from recipients with BN-negative kidneys or hearts were also found negative in tests for haemagglutinating antibodies

The findings with sera from recipients with BN-positive kidneys or hearts varied considerably

Fig 3 BN negative (AS × BN)F2 kidney 471 after grafting to an AS recipient Signs of a chronic nephritis with dilated tubules, filled eosinophilic colloid material H & E × 56

Fig 4 AS remaining kidney 12 months aft unilateral nephrectomy was performed High de of chronic nephritis with tubular atrophy tubules dilated and filled with eosinophilic c Interstitial tissue infiltrated with chronic infl matory cells H & E × 56

Fig 5 Day 6 biopsy from a BN positive (Fische BN)F2 kidney grafted to a Fischer recipient Mo nuclear cell infiltration and tubular necrosis H × 56

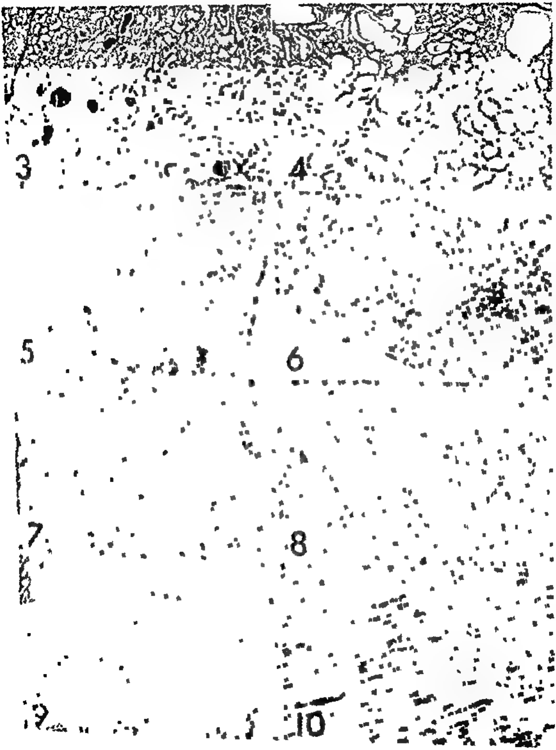
Fig 6 Day 6 biopsy from a BN negative (Fis × BN)F2 kidney grafted to a Fischer recipi Very slight degree of mononuclear cell infiltra and no tubular necrosis H & E × 56

Fig 7 BN positive (Fischer × BN)F2 kid rejected by a Fischer recipient day 12 Mo nuclear cell infiltration, tubular necrosis and obl ration of the glomeruli with eosinophilic mater H & E × 140

Fig 8 BN negative (Fischer × BN)F2 kid grafted to a Fischer recipient at day 273 af operation, showing no evidence of rejection H & × 140

Fig 9 BN positive (Fischer × BN)F2 heart rejec by a Fischer recipient day 12 Mononuclear infiltration and muscular necrosis H & E × 1

Fig 10 BN negative (Fischer × BN)F2 he grafted to a Fischer recipient at day 285 aft operation showing no evidence of rejection H & × 140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In some of the sera haemagglutinating antibodies could be demonstrated one week after transplantation up to a titre of 1 243 In some of these recipients the antibodies disappeared again after 2 weeks, in others they persisted for 3–4 weeks

In sera from other recipients no antibodies were detected after 1 week, but turned up after 2–3 weeks, and finally in some of the recipients no antibodies were found at any stage

No correlation between rejection times and presence of antibodies was apparent

No conclusion could therefore be drawn from the studies of haemagglutinating antibodies, except that these never appeared in recipients of kidneys or hearts from donors which didnot carry the foreign Ag-B allele from the BN strain

DISCUSSION

In Simonsen's early postulate (15) that kidney transplantation in man was determined by particularly strong antigens of a single histocompatibility locus, the notion was inherent that all the weaker antigens led to tolerance instead of immunity in the conditions given by the heavy antigenic load of organ grafting facilitated by immunosuppression He and his coworkers have later questioned whether immunosuppression is at all needed to secure permanent survival of organ grafts provided the donor carries exactly the same alleles of the strong locus as the recipient Their preliminary findings in rats (4, 13) suggested that immunosuppression might not be needed, and some results from other laboratories have pointed towards the same conclusion (9, 20, 21), while others have not (7)

The present work was designed to test the one locus hypothesis for kidney and heart grafting in rats The simple predictions from that hypothesis were clearly that organs from F2 donors to parental recipients should always be rejected when they carried the Ag B allele of the opposite parent, but should be accepted when they did not The expected rejection occurred with all of 25 heart grafts and with 17 out of 18 kidney grafts The remaining kidney was probably rejected, too, but only after 197 days On the other hand, the expected long term, and probably permanent acceptance of the graft occurred with 19 out of 21 hearts and with all of 16 kidneys One heart was clearly rejected after 62 days and another possibly after 244 days Skin grafts from the same donors to separate parental strain recipients were as expected all rejected, usually after 2–2½ weeks

The findings in this study are, with respect to kidney grafts, in accordance with the observations of *White & Hildemann* (20, 21) and *Mahabir et al* (9) Both groups of investigators demonstrated long term survival of kidney allografts in unmodified inbred rats when the donors and recipients were matched at the Ag-B histocompatibility locus while differing at numerous minor histocompatibility loci and rejecting skin grafts acutely *White & Hildemann* used the Fischer to Lewis combination, and *Mahabir et al* the (Fischer × Lewis)F1 to Lewis combination

The long-term survival of the heart grafts from BN negative donors does, however, contrast with the observation of *Freeman & Steinmuller* (7) who showed that Fischer heart grafts were rejected by Lewis rats nearly as rapidly as Fischer skin grafts They ascribed the difference in survival times of Fischer kidneys and Fischer hearts in Lewis recipients to hypothetical tissue- or organ specific histocompatibility antigens

Also *Barker & Billingham* (1) found acute rejection when hearts were grafted from Fischer to Lewis rats, but greatly prolonged survival when hearts from (F × L)F1 × L back cross donors were grafted to Lewis recipients

In all of these studies involving the strain combination of Fischer with *Lewis* there remains some doubt as to the real meaning of the alleged Ag-B identity True enough *Elkins & Palm* (6, 10) have found the two strains identical by serotyping But this fact might possibly reflect a situation analogous to the so called HL-A identical unrelated human beings who nevertheless nearly al

ways show stimulation in MLC, although at a level of reactivity usually lower than that in unrelated which do not type identical (19) Whatever may be the truth about the Ag B identity of these two strains there can of course be no questioning the published fact that Fischer heart grafts are rejected acutely while kidney grafts enjoy a greatly prolonged survival In the comparison of heart with kidney grafts a similar difference has clearly not been found in our studies On the contrary if any difference, we see a trend in the opposite direction

Only a single exception can be seen to support the finding reported by *Freeman et al* This is the case in Table 1 Group I where the heart stopped beating after 62 days whereas the kidney recipient was killed in good condition on day 308 The heart showed all characteristic signs of rejection which must presumably have been caused by an exceptional constellation of weak BN histocompatibility antigens in this particular donor But then it causes some difficulty to explain why the kidney from the same donor was not rejected The possibility of the heart being more sensitive than the kidney to incompatibility caused by the weaker loci will have to be considered

On the other hand, we found in our previous report on grafting to AS recipients from back-cross hybrids with AS2 (4) an unexpected greatly prolonged survival of hearts grafted across a strong Ag B barrier which contrasted with rapid rejection of the kidney grafts in most cases of the same combination

The tendency of grafted hearts to survive better than kidneys, when grafted across an Ag B barrier is also demonstrated to a smaller extent in this study in the AS-BN combination though not in the Fischer BN combination where there was no difference

The most striking and convincing inconsistency with the predictions from the onelocus hypothesis so far found is *Salaman's* (14) finding in kidney grafting from AS → AS2 These strains are known to differ at the major Ag B locus and yet the kidney grafts function usually for months as we have also been able to confirm in a recent study (3 18)

Salaman suggested that long term survival of organs grafted across an Ag B barrier was due to development of immunological tolerance while we are now more inclined to assume an enhancement mechanism At least it is clear from graft versus host assays performed with spleen and lymph node cells from long time surviving AS2 recipients of AS kidneys that these recipients are in fact far from being tolerant to AS antigens (3)

So in conclusion while incompatibility at the Ag B locus does not invariably lead to rejection of either kidney or heart grafts acute rejection is certainly the common rule On the other hand it has so far been very difficult in our material to find exceptions to the rule that compatibility at the Ag B locus leads to permanent acceptance

This latter conclusion is supported also by the findings in an unpublished small scale study of heart grafting in wild rats Among wild rat siblings delivered by caesarian section and nursed by laboratory rat foster mothers we found 2 pairs which failed to stimulate each other in MLC tests On the strength of this criterion of Ag B identity heart grafting was then performed in both pairs from one rat to the other Unfortunately one of the recipients was killed by accident 2 weeks after the transplantation but the heart showed no histological sign of rejection The other grafted heart is still functioning after more than 6 months

Other studies in progress concern kidney and heart grafting from LEW BN to BN This combination of congenic strains developed by *Stark et al* (16) are identical at the Ag B locus while differing at a minimum of 14–15 minor loci (17) Till now all grafts have been perfect takes but the time of observation is still too short to allow definitive conclusions

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IMMUNOGLOBULINS IN BOVINE LACHRYMAL FLUID

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By immunochemical quantitation it has been shown that IgA is the main immunoglobulin in bovine lachrymal fluid, constituting approximately 40 per cent of the total protein content. The concentrations of IgM and IgG 1 were considerably lower. IgG-2 usually occurred in concentrations too low to be quantitated. While lachrymal IgA levels varied mainly independently from the IgA serum levels there was a significant positive correlation between the lachrymal and serum levels of both IgM and IgG-1, indicating that the bulk of these immunoglobulins may be derived from the plasma.

The immunoglobulins in external secretions bathing the mucous membrane surfaces of the organism may be of great importance in resistance to infection. In the bovine several features of the immunoglobulins are consistent with the general pattern established for immunoglobulins of other mammalian species but in addition there are some peculiar traits of the exocrine immune system which seem to be specific for ruminants. The most striking feature is the high content of IgG-1 compared with IgG 2 and IgA in the colostral secretion. During the last decade considerable interest has been given to colostral immunoglobulins, whereas the study of immunoglobulins in other external secretions of this species has just recently been initiated (*Mach et al.* 1969, *Porter & Noakes* 1970, *Mach & Pahud* 1971, *Curtain et al.* 1971).

The present study on the immunoglobulin composition of normal lachrymal fluid is part of a series of experiments which are primarily designed to elucidate the immune phenomena of the conjunctival mucosa. Such experiments may be useful for the understanding of the course of local infections on this mucous membrane, but some general traits of the exocrine immune system may also be revealed. The lachrymal fluid is a representative external secretion possessing obvious advantages. Thus, it is easily obtained from the living non anaesthetized animal and it is accessible in a highly pure form.

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MATERIALS AND METHODS

Collection of Materials

Animals. The present study comprised 20 normal female animals of the Red Danish Milkbreed. The age of the animals appears from Table 4.

Serum. Blood samples were drawn from the jugular vein. Serum was stored at −20°C.

Lachrymal fluid. From each animal one ml of lachrymal fluid was collected from one eye. The fluid was sucked from the lower conjuctival sack by means of a 1,000 µl Carlsberg pipette. Immediately after collection the samples were frozen by means of solid carbon dioxide and brought to

the laboratory where they were stored at –20°C Before use the fluid was centrifuged in capillary tubes at 23,000 g for 15 minutes to remove any insoluble material

Chromatographic Methods

Ion exchange chromatography was performed using DEAE-*Sephadex* A–50 *(Pharmacia, Uppsala, Sweden)* Before packing the column the ion exchanger was allowed to swell in a phosphate buffer *(0 02 M, pH 8 0) for 24 hours* A continuous and concave ionic strength gradient was produced by mixing two phosphate buffers in a *Varigrad (Boskamp Gerätebau* KG) The ionic strength of the initial buffer was the same as that of the buffer used for swelling, *i e* 0 02 M, pH 8 0 and the ionic strength of the limit buffer was 0 6 M, pH 8 0 The shape of the gradient was calculated according to *Peterson & Sober* (1959) The protein content in the effluent was recorded at 280 nm in a LKB 8300 *Uvicord (LKB product AB, Stockholm, Sweden)* Before application to the column the sample was dialysed at 4°C for 24 hours against *the initial buffer*

Gel filtration chromatography was performed on *Sephadex* G 200 Superfine in 0 1 M tris (hydroxymethyl) aminomethane + 1 0 M NaCl, pH 8 0 The protein concentration in the effluent fluid was recorded as described above

Preparation of Immunoglobulins

IgG–1 and IgG 2 were isolated from serum by DEAE-*Sephadex* chromatography according to *Nansen (1970) i e* IgG–2 was obtained from normal serum as the break through peak, while IgG–1 was isolated from an IgG–2 deficient serum at a buffer molarity of approximately 0 05 M *(pH 8 0)*

IgA was isolated from lachrymal fluid pooled from 15 normal adult cows (RDM) After concentration by vacuum dialysis three ml containing 115 mg protein were applied to a DEAE-*Sephadex* column Three grams, dry weight of the ion exchanger were used to prepare a 1 7 × 25 cm column As evidenced by immunoelectrophoresis and single radial immunodiffusion IgA was eluted in the second peak of the chromatogram (Fig 2) Fractions containing IgA were concentrated by vacuum dialysis and subsequently filtered on a *Sephadex* G–200 column The front half of the first protein peak was rechromatographed on the same column and one symmetrical peak was obtained The material was analysed by double diffusion in agar gel against rabbit anti bovine lachrymal fluid and rabbit anti bovine IgA By each antiserum only one precipitation line was developed The two lines were completely confluent

IgM was isolated from serum pooled from two hyperimmunoglobulinaemic cows (RDM) Fifty ml serum were filtered on a column of *Sephadex* G–200 with bed dimensions 7 × 75 cm As judged by immunoelectrophoresis IgM was eluted in the first peak of the chromatogram The front third of this peak was concentrated by vacuum dialysis and in order to purify IgM further the material was rechromatographed twice on *Sephadex* G-200 columns IgM was not obtained in a completely pure state A very slight contamination with a protein—probably identical with α_2M—was recorded by immunoelectrophoresis and by double diffusion in agar gel The amount of this latter protein was too small to be revealed by paper electrophoresis since all detectable protein was confined to the gamma area

Preparation of Antisera

Rabbit anti bovine serum was prepared by immunization with serum pooled from 22 normal RDM cows

Anti bovine lachrymal fluid was prepared by immunization with a pool of lachrymal fluid from 15 normal adult cows

The preparation of monospecific antisera to bovine IgG–1 and IgG 2 was performed as previously described by immunizing rabbits with IgG–1 or IgG 2 and absorbing exhaustively with IgG–2 and IgG–1 resp *(Nansen* 1970) The anti bovine IgG–2 was not directed against the entire IgG–2 antigenic spectrum A few sera did not react with this antiserum although immunoelectrophoresis using the anti bovine serum in fact revealed small quantities of IgG–2 (*Table 4*) This phenomenon has also been noted by *Nansen* (1970) and *Aalund & Kruse* (1971) This means that the absolute IgG–2 concentrations listed in *our tables may be more or less underestimated and should be* critically evaluated On the other hand the quantitative data on IgG–2 are of value when the relation between lachrymal and serum levels *is considered.* Rabbit anti bovine IgA and IgM were produced according to *Nansen et al* (1971) by immunizing rabbits with agar gel precipitates followed by absorption to obtain monospecificity Rabbit anti bovine albumin was obtained by immunization with salt precipitated serum albumin *(Serum Albumin vom Rind* trocken, *"reinst" (Behringwerke AG))*

Immunochemical Procedures

Immunoelectrophoresis was performed by the micromethod of *Scheidegger* (1955) Double diffusion in agar gel was performed according to *Ouchterlony* (1958)

Immunochemical quantitation of protein was performed by the single radial immunodiffusion method described by *Mancini et al* (1965) and slightly modified by *Jensen* (1966) The error involved in this procedure was evaluated by application of one particular standard on all agar

plates The variations for each of the proteins are listed in Table 1

TABLE 1 *Repeated Protein Determinations on One Serum*

Protein	Number of determinations	Coefficient* of variation
Albumin	41	10.3
IgG-1	16	5.5
IgG-2	25	20.8
IgA	11	6.7
IgM	12	5.1

* Standard deviation expressed as percentage of the mean

The protein concentration of standard solutions of IgG-1, IgG-2 and albumin was determined by a micro Kjeldahl method using the factor 6.25 for the conversion of nitrogen to protein The concentration of the standard samples of lachrymal IgA and of serum IgM was measured by absorption at 280 nm in a *Beckman DB G Spectrophotometer* since the protein quantities were too small to be determined by the micro Kjeldahl method The extinction coefficients $E^{1\%}_{280} = 13.4$ for IgA and $E^{1\%}_{280} = 13.3$ for IgM were used as conversion factors for the calculation of protein values (*Schultze & Heremans* 1966)

RESULTS

Immunoelectrophoresis using bovine lachrymal fluid as antigen and anti bovine lachrymal fluid as antiserum revealed 10–15 precipitation lines Some of the proteins in lachrymal fluid are apparently identical with proteins found in serum while others seem to be unique to lachrymal fluid (Fig 3) The elution zones of the immunoglobulins after *Sephadex* G–200 chromatography of concentrated lachrymal fluid are indicated in Fig 1 In Fig 2 is shown the elution pattern after chromatography on DEAE *Sephadex* A–50

The immunoglobulin, albumin and total protein levels of the lachrymal fluid are shown in Tables 2 and 3 In Table 2 the results are given as mg per 100 ml of lachrymal fluid, and in Table 3 as mg per 100 mg of total protein Table 4 shows the immunoglobulin concentrations of serum

Table 5 shows the ratios of immunoglobulins to albumin in serum as well as in lachrymal fluid By comparing these ratios for the individual immunoglobulin in lachrymal fluid and in serum it is possible to determine whether there in lachrymal fluid is a relative predominance of a certain immunoglobulin It is presumed that albumin in lachrymal fluid has entered this fluid as the result of a passive epithelial leakage If the ratio of immunoglobulin to albumin in lachrymal fluid is higher than that of serum, this may either be due to a selective transport from serum to lachrymal fluid or to a local synthesis of the immunoglobulin concerned or both

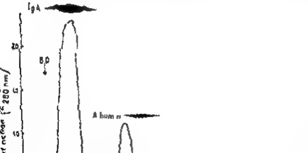

Fig 1 Sephadex G–200 chromatogram of concentrated bovine lachrymal fluid Column dimensions 2.5 × 34 cm flow rate 7.8 ml/h, sample 1.4 ml with a protein content of 35.1 mg/ml Fractions 3.0 ml Elution patterns of immunoglobulins were determined by single radial immunodiffusion Elution position for Blue dextran (BD) is indicated by the arrow

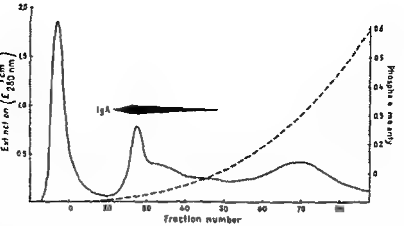

Fig 2 DEAE-Sephadex A 50 chromatogram of concentrated bovine lachrymal fluid The solid line indicates the protein content of the effluent The dotted line shows the increment in the phosphate molarity of the gradient as indicated on the ordinate axis to the right Column dimensions 1 7 × 25 cm flow rate 20 ml/h sample 5 0 ml with a protein content of 35 1 mg/ml Fractions 4 0 ml Elution pattern for IgA was determined by single radial immunodiffusion

The total protein content of lachrymal fluid was on an average 640 mg per 100 ml No significant correlation was found between total protein levels in serum and lachrymal fluid ($P > 0.05$ where P represents the probability that $r = o$) The albumin levels in the lachrymal fluid were very low compared with those of the serum Thus, the average concentration was 595 times lower than the average serum level

IgA is quantitatively the predominating immunoglobulin in lachrymal fluid (average 250 mg/100 ml) and constitutes on the average 41 per cent of the total protein content There was a significant correlation between the IgA levels of serum and lachrymal fluid when expressed as mg/100ml ($0.05 > P > 0.01$ Fig 4) but not when expressed as mg/100 mg protein ($P > 0.05$) The most striking feature of the lachrymal immunoglobulin levels was that the IgA to albumin ratio was appr 13 000 times higher in lachrymal fluid than in serum (Table 5)

The IgG–1 level in lachrymal fluid was higher than expected if a non selective diffusion was the only transfer mechanism Whereas the IgG–1 to albumin ratio in serum was 0 5, the ratio was 3 0 in lachrymal fluid The concentration of IgG–1 in lachrymal fluid was positively correlated with the concentration of the same immunoglobulin in serum (Expressed either as mg/100 ml $0.05 > P > 0.01$ (Fig 5) or as mg/100 mg protein $P < 0.01$)

The IgG–2 levels in lachrymal fluid were so low that they only could be determined in a few animals (Table 2) In these animals the IgG–2 to albumin ratio was approximately twice as high as the same ratio in serum In all cases in which the IgG–2 levels were below the lowest limit of sensitivity i e 0 6 mg/100 ml the average IgG–2 to albumin

Fig 3 Immunoelectrophoresis of bovine lachrymal fluid (top well) and bovine serum (bottom well) Antibody trench Rabbit anti bovine lachrymal fluid Anode to the right

TABLE 2 *Protein Levels (mg/100 ml) in Lachrymal Fluid of 20 Animals*

J No	Total protein	Albumin	IgG-1	IgG-2	IgA	IgM
2	590	5 65	18 9	< 0 6	282	7 9
34	350	2 23	4 4	< 0 6	213	10 4
423	380	2 05	7 4	< 0 6	234	10 7
4	630	5 45	20 9	< 0 6	267	12 0
465	650	2 50	5 1	< 0 6	222	8 9
262	470	2 60	6 5	1 6	144	13 5
476	530	5 25	15 9	N D	221	15 2
19	810	27 9	22 7	5 2	272	106
49	490	4 13	11 5	1 8	128	21 6
50	470	9 78	10 8	< 0 6	230	36 7
491	570	3 58	21 2	< 0 6	309	121
32	660	4 40	12 9	2 1	213	27 5
29	720	7 33	11 7	< 0 6	309	35 5
319	690	4 75	12 5	2 5	309	11 4
453	510	3 43	10 3	< 0 6	227	37 1
437	1210	3 45	13 7	1 4	476	116
27	530	7 70	22 8	< 0 6	199	37 9
457	1100	3 33	19 3	< 0 6	223	37 2
11	620	6 08	20 2	< 0 6	246	72 6
489	800	2 40	9 3	N D	272	16 8
Mean	639	5 70	13 9		250	37 8
S D	220	5 6	6 0		72	37

S D Standard deviation
N D Not determined

TABLE 3 *Protein Levels (mg/100 mg Protein) in Lachrymal Fluid of 20 Animals*

J No	Albumin	IgG-1	IgG-2	IgA	IgM
2	1 0	3 2	< 0 1	47 8	1 3
34	0 6	1 3	< 0 2	60 8	3 0
423	0 5	1 9	< 0 2	61 5	2 8
4	0 9	3 3	< 0 1	42 4	1 9
465	0 4	0 8	< 0 1	34 2	1 4
262	0 6	1 4	0 3	30 6	2 9
476	1 0	3 0	N D	41 7	2 9
19	3 4	2 8	0 6	33 5	13 1
49	0 8	2 3	0 4	26 1	4 4
50	2 1	2 3	< 0 1	48 9	7 8
491	0 6	3 7	< 0 1	54 2	21 2
32	0 7	2 0	0 3	32 3	4 2
29	1 0	1 6	< 0 1	42 9	4 9
319	0 7	1 8	0 4	41 8	1 7
453	0 7	2 0	< 0 1	44 5	7 3
437	0 3	1 1	0 1	39 3	9 6
27	1 5	4 3	< 0 1	37 5	7 2
457	0 3	1 8	< 0 05	20 5	3 4
11	1 0	3 3	< 0 1	39 [illegible]	11 7
489	0 3	1 2	N D	34 0	2 1
Mean	0 9	2 3		40 9	5 7
S D	0 7	1 0		11	5

S D Standard deviation
N D Not determined

TABLE 4 *Age and Serum Protein Levels (mg/100ml) of 20 Animals*

J No	Age (years)	Total serum protein	Albumin	IgG-1	IgG 2	IgA	IgM
2	4	7780	3100	2530	*	40 0	622
34	4	7680	3740	900	1380	12 4	747
423	5	7720	3490	1370	1200	20 4	379
4	4	7490	3390	2600	741	36 1	573
465	4	7440	3710	1190	61	9 5	443
262	2	6920	3150	1460	439	7 7	734
476	4	7680	3830	1820	*	27 7	771
19	3	7030	3090	1160	675	6 7	751
49	2	7210	3180	1670	823	13 2	833
50	2	7180	3190	1430	100	6 4	846
491	4	6850	3230	1710	133	16 0	829
32	2	7390	3610	1660	853	22 1	736
29	2	7120	3700	900	481	23 0	553
319	7	7150	3470	1470	1040	17 6	383
453	10	7230	3530	1230	458	9 0	542
437	6	7330	3400	1310	598	27 8	1190
27	2	6910	2850	1850	172	7 7	801
457	5	6610	3510	1310	*	11 0	589
11	2	6420	2610	1440	50	0 6	1200
489	4	7200	3760	1310	851	19 2	549
Mean		7222	3391	1516		16 7	700
S D		362	322	443		10	290

S D Standard deviation
* Small quantities detectable by immunoelectrophoresis using anti total bovine serum

ratio in lachrymal fluid was lower than the average serum ratio

IgM was found in higher concentrations than IgG-1 in the lachrymal fluid The average concentration was 37 8 mg/100 ml equivalent to 5 7 mg/100 mg protein The IgM to albumin ratio was 41 times higher in lachrymal fluid than in serum The correlation between the concentration of IgM in serum and lachrymal fluid was statistically significant (Expressed as mg/100 ml $P < 0.01$ (Fig 6) or as mg/100 mg protein $0.05 > P > 0.01$)

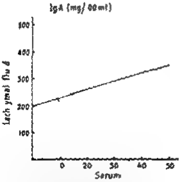

Fig 4 The relation between the concentrations of IgA in serum and lachrymal fluid The equation for the regression line is $y = 3.02x + 200$ The coefficient of correlation is $r = 0.42$ $0.05 > P > 0.01$

DISCUSSION

Several problems are involved in the quantitation of immunoglobulins in lachrymal fluid Thus it has been observed in man that the protein concentration decreases with increasing flow rate (*Josephson* & *Weiner* 1968) Hence the absolute concentrations are only of value when the collection conditions are known and defined Determination of the flow rate of lachrymal fluid in adult non anaesthetized cows is practically impossible and a standard collection procedure is necessary Therefore in the present investigation

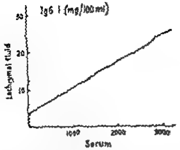

Fig 5 The relation between the concentrations of IgG-1 in serum and lachrymal fluid The equation for the regression line is $y = 0.007x + 3.25$ The coefficient of correlation is $r = 0.52$, $0.05 > P > 0.01$

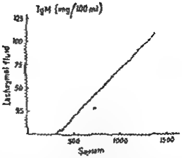

Fig 6 The relation between the concentrations of IgM in serum and lachrymal fluid. The equation for the regression line is $y = 0.10x - 35.4$ The coefficient of correlation is $r = 0.62$ $P < 0.01$

the same person collected under similar conditions a constant volume (1.0 ml) from each animal However, undecipherable variations may still exist and it would therefore be most reasonable to state the results as mg/100 mg protein since it may be assumed that the protein content of bovine lachrymal fluid decreases with increasing flow rate and that the concentrations of all immunoglobulins fall proportionally to the total protein content

Another problem in connection with the quantitation of immunoglobulins in external secretions is the choice of IgA standard In the present work lachrymal IgA was used

TABLE 5 *Ratios of Immunoglobulins to Albumin in Serum and Lachrymal Fluid*

J No	Serum			Lachrymal fluid		
	IgG-1 / albumin	IgA / albumin	IgM / albumin	IgG-1 / albumin	IgA / albumin	IgM / albumin
2	0.8	0.015	0.2	3.3	49.9	1.4
34	0.2	0.003	0.2	2.0	95.5	4.6
423	0.4	0.006	0.1	3.6	114	5.2
4	0.8	0.010	0.2	3.8	49.0	2.2
465	0.3	0.003	0.1	2.0	88.8	3.6
262	0.5	0.002	0.2	2.5	55.4	5.2
476	0.5	0.007	0.2	3.0	42.1	7.9
19	0.4	0.002	0.2	0.8	9.7	3.8
49	0.5	0.004	0.2	2.8	31.0	5.2
50	0.4	0.002	0.3	1.1	23.5	3.8
491	0.5	0.005	0.3	5.9	86.3	33.8
32	0.3	0.007	0.2	2.9	48.4	6.3
29	0.2	0.006	0.1	1.6	42.2	4.8
319	0.4	0.005	0.1	2.6	65.1	2.4
453	0.3	0.003	0.2	3.0	66.2	10.8
437	0.4	0.008	0.3	4.0	138	33.6
27	0.6	0.003	0.3	3.0	25.8	4.9
457	0.4	0.003	0.2	5.8	67.6	11.2
11	0.6	0.0002	0.5	3.3	40.5	11.9
489	0.3	0.005	0.1	3.9	113	7.0
Mean	0.5	0.0049	0.2	3.0	62.6	8.2
S D	0.2	0.003	0.1	1	34	9

S D Standard deviation

This entails overestimation of the serum IgA values *Brandtzaeg et al* (1970) have found that the monomer (7S) fraction is about three times overestimated when a polymer IgA standard is used So, in reality the serum IgA values found in this investigation are even lower than those listed in Table 4

The present study has provided quantitative data on the immunoglobulin classes in bovine lachrymal fluid and related these to the corresponding serum levels of the same individual The major immunoglobulin in lachrymal fluid was found to be IgA The levels in the present study correspond with the levels recently published by *Mach & Pahud* (1971) The serum IgA levels were low compared with serum IgA in man Similar low levels in the bovine have been reported by *Porter & Noakes* (1970) and by *Mach & Pahud* (1971) From the regression line between IgA in serum and lachrymal fluid it may be inferred that the bulk of lachrymal IgA is not derived from the blood Thus, a serum IgA concentration of 0 mg/100 ml corresponds to a lachrymal IgA concentration of 200 mg/100 ml (Fig 4) Moreover, the many times higher ratio of IgA to albumin in lachrymal fluid than in serum (average 13,000) suggests that by far the most IgA in lachrymal fluid is locally synthesized

While most work in the bovine species up to now has been focused on the immunochemical properties of IgA *Morein* (1970) has shown that antibody activity against *Parainfluenza-3* virus resides in IgA Recently *Pedersen et al* (1971) demonstrated that vaginal mucous antibody activity to *Vibrio foetus* was partly residing in IgA From the immunoglobulin pattern of lachrymal fluid it may be assumed that a predominance of the broad spectrum of antibody activities reside in the IgA class

The IgM concentrations in lachrymal fluid were consistently lower than those of IgA but on the other hand somewhat higher than the IgG–1 concentrations This latter finding is not consonant with the data presented by *Mach & Pahud* (1971) since in their experiments IgG–1 levels constantly exceeded IgM levels in lachrymal fluid as well as in other body fluids Likewise our IgM serum levels were also high compared with those found by *Mach & Pahud* and consistently higher than those usually found in human serum. It is not possible on the basis of our experiments to define conclusively the origin of lachrymal IgM The significant correlation between the concentrations of IgM in serum and lachrymal fluid may possibly indicate the presence of preferentially plasma derived IgM molecules in lachrymal fluid It should be noted that in man there seems to exist a selective glandular transfer of IgM, and unlike the IgA system this secretory IgM is apparently similar to its *19S* counterpart in serum (*Brandtzaeg* 1968 *Brandtzaeg et al* 1970)

One very conspicuous finding in the present study was the demonstration of high IgG–1 concentrations compared with IgG–2 concentrations in the lachrymal fluid This is comparable with the IgG concentrations of colostrum The main immunoglobulin of this secretion is IgG–1, whereas IgG–2 is present in only insignificant amounts (*Murphy et al* 1965, *Pierce & Feinstein* 1965, *Aalund* 1968 *Kickhofen et al* 1968 *Mach & Pahud* 1971) IgG–1 appears to be derived from the plasma as indicated by radio isotopical studies (*Blakemore & Garner* 1956, *Larsen & Gillespie* 1957, *Garner & Crawley* 1958 *Dixon et al* 1961) In fact, immunohistological studies (*Dixon et al* 1961) and studies using tissue culture with incorporation of labelled amino-acids (*Mach & Pahud* 1971) provide little or no evidence for a local synthesis of IgG–1 by the mammary gland The presence of IgG–1 in the lachymal fluid and other exocrine secretions may at least partly be explained by a similar direct and selective transfer from the serum Thus radio isotopical studies by *Curtain et al* (1971) point to some degree of selectivity in transport of IgG–1, compared with IgG–2 through the mucosae of the gut and the respiratory system It should be noted that these findings are in agreement with recent experiments in our laboratory which reveal

consistently higher ratios of radio iodinated IgG-1 to IgG-2 in the lachrymal fluid than in the plasma (*Pedersen* 1972). However, in addition to the transfer of immunoglobulin from the plasma studies by *Mach & Pahud* (1971) and *Curtain et al* (1971) suggest that the majority of IgG-1 in secretions other than colostrum is locally synthesized. The relative participation of local synthesis and transfer of immunoglobulin from the plasma into the lachrymal fluid is unknown but the findings in the present study that the lachrymal concentration of IgG-1 varies in direct proportion with the serum level possibly indicate a predominance of plasma-derived molecules. This contrasts with lachrymal IgA which varies mainly independently from the serum concentration.

Finally, the present results confirm the impression given by recent studies (*Mach & Pahud* 1969, 1971) that cattle, and possibly other ruminants, are unusual in possessing different secretory immune systems, *i.e.* an IgA and an IgG-1 system. Locally synthesized IgA plays a minor quantitative role in colostrum but it is the predominant immunoglobulin class in other external fluids. IgG-1, on the other hand, is the main immunoglobulin of colostrum, almost exclusively transferred from the plasma by a selective mechanism. In the other secretions this immunoglobulin may be partly plasma-derived and partly locally produced. IgM is not the main immunoglobulin in any external secretion, but compared with *e.g.* IgG-2 it is present in substantial quantities, probably due to a selective transfer.

In the study of local immune mechanisms the lachrymal gland and conjunctiva possess obvious advantages. 1) The lachrymal fluid is readily accessible in a highly pure form. 2) Pathological changes of *e.g.* the conjunctival mucosa can be directly examined. 3) The immune response to local infections can be studied in the one eye, using the other eye of the same animal as a control. It has been shown that infection with *Moraxella bovis*, a causative micro-organism of infectious keratoconjunctivitis in cattle may provide a useful model for experiments on local infection and immune phenomena (*Pedersen* 1970). Studies on the mechanism of appearance of immunoglobulin classes in response to infection with *M. bovis* are in progress in our laboratory (*Pedersen* 1972).

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ASSOCIATION OF METHICILLIN RESISTANCE TO PRODUCTION OF ENTEROTOXIN B AND OTHER FACTORS IN COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI

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Methicillin resistance in *S albus* strains isolated from clinical sources was of two types. One type was heterogeneous and highly resistant the other non heterogeneous and less resistant. Of 80 methicillin resistant strains of *S aureus* 82 5 per cent were enterotoxin B procedures but none of the methicillin resistant strains of *S albus* produced enterotoxin B Experiments to determine whether any linkage existed between enterotoxin B nuclease and coagulase production showed that all three characters could vary independently

In routine bacteriology the pathogenic *Staphylococcus aureus* is usually defined by its capacity to produce coagulase or nuclease (7) There is however growing evidence that coagulase negative staphylococci '*Staphylococcus albus*' can also be pathogenic for man (9 18) This group of bacteria is rather composite and according to Baird Parker constitutes at least five types (2 3 19) As with other bacteria resistant strains have appeared and both in London (9) and Boston (18) 10 per cent of the coagulase negative staphylococci were reported to be resistant to methicillin

In *S aureus* methicillin—resistance has been suggested to be associated with enterotoxin B—production (11 14) The purpose of this work was to study whether this was true also for methicillin—resistant *S albus* Wild strains as well as laboratory mutants were analysed

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MATERIALS AND METHODS

Strains

1 80 coagulase positive methicillin—resistant staphylococci have been bacteriologically analysed 74 of the strains were supplied from twelve laboratories throughout the world Jevons Colindale, London England Dyke Oxford England Churcher Plymouth, England Turner Liverpool England Brandis Gottingen Germany Pulverer Cologne Germany Rosendal Copenhagen Denmark Kayser Zurich Switzerland Rédai Hungary Bulger Washington USA Sabath Boston USA Rountree Sydney Australia 6 are type strains representing outbreaks in 5 Swedish hospitals Kallings, Stockholm Kamme Lund Kjellander Orebro Hallander Vasterås, Dornbusch Uppsala Four strains—one from Gottingen BG 6969 one from Cologne PK 4516 one from

Uppsala DU 4916 and one from Vasterås HV 1779—were used for elimination studies

2 30 coagulase—negative methicillin—resistant staphylococci were isolated from infected wound and urine at the departments of clinical bacteriology in Uppsala and Vasterås, Sweden All fermented glucose anaerobically (2, 3)

Antibiotic Sensitivity Tests

Methicillin—resistance was determined in a plate dilution test Resistance was defined as growth in the presence of 12 5 μg of methicillin per ml after 48 hours at 30° C with an inoculum of 0 1 ml of an 18 hours broth culture (13)

A disc diffusion test was used for all other antibiotics

Assay of Toxins and enzymes

Enterotoxin [illegible] haemolysins, lipase and coagulase were determined as described by *Hollander* (12)

DNase was determined by growing bacteria on DNA agar plates (BBL) or filling agar cups in the same medium with supernatant fluid from Casman culture (12) After 24 hours at 37° C undigested DNA was developed by flooding the plates with 1 N sulphuric acid

Phosphatase was determined according to the method of *Barber and Kuper* using phenolphtalein phosphate agar (4)

Anaerobic mannitol was determined by stab inoculation into phenolmannite agar (9)

Phage typing was performed according to *Blair & Williams* (5) with the standard phage set recommended at the Moscow meeting 1966

Mutagenesis

1 *Ethyl Methane Sulphonate* (EMS) 0 1 ml of EMS was added to 2 ml of a 4 hours culture in brain heart infusion (BHI) broth mixed and shaken at 37° C for 30 minutes (15) After centrifugation the bacteria were resuspended in BHI and incubated overnight at 37° C

2 *1 methyl—3 nitro—1 nitrosoguanidine* (MNG) Centrifuged bacteria of an overnight culture in BHI were diluted to a concentration of appr 2×10^7 cells/ml in sterile 0 9 per cent saline containing 200 μg/ml MNG The suspension was then incubated at 30° C for 20 minutes diluted in saline and plated (1)

3 *Ethidium bromide* (Etbr) The strain to be tested was inoculated into BHI broth containing 3 μg/ml of Ethidium bromide and incubated over night at 37° C (6)

4 *Ultra—violet irradiation* 4 hours cultures in BHI were irradiated in an open petri—dish 20 cm from an uv lamp (Phillips TUV 6 W 57416 F/40) whilst being shaken gently to give a survival of c 0 001 *per cent* This was usually about 15 minutes Then the undiluted cultures were plated immediately

Detection of DNase Negative Mutants

The treated cultures of bacteria were plated in 0 1 ml aliquots at an appropriate dilution to give c 100 colonies/plate onto DNase test agar (BBL) After 24 hours at 37° C the bacteria were replicated onto nutrient agar plates containing *100 μg* ml methicillin (NA met) Both sets of plates were incubated for a further 24 hours (DNA agar at 37° C and NA met agar at 30° C) The DNA agar plates were then flooded with 1 N sulphuric acid and the individual colonies examined. Colonies which did not produce a zone of clearing were presumed to be DNase negative and subcultured from the NA met plate onto a DNA plate for confirmatory testing

RESULTS

Resistance Pattern

All 80 strains of *S aureus* were multiresistant and showed the heterogeneous type of resistance to methicillin which has been described in many papers *i e* $1/10^4$–$1/10^6$ of the organisms growing in the presence of 400 μg/ml methicillin (13 17) After cloning on methicillin plates the strains acquired a higher resistance which remained unchanged for several generations *before* they slowly segregated The coagulase—negative staphy

TABLE 1 *Resistance Patterns of Methicillin Resistant Staphylococci*

		No of strains	Number resistant for				
			penicillin G	erythromycin	streptomycin	tetracyklin	chloramphenicol
Coagulase positive		[illegible]	80	55	79	78	25
Coagulase negative	type 1	21	21	5	21	19	11
	type 2	9	0	0	[illegible]	[illegible]	0

TABLE 2 *Production of Enterotoxin B and Other Factors by Methicillin—Resistant Staphylococci*

		No of strains	Positive for					
			DNase	ent B	tween	α hem $>1/8$	β hem $>1/8$	δ hem $>1/8$
Coagulase positive		80	80	66	9	70	53	54
Coagulase negative	type 1	21	0	0	21	10	0	10
	type 2	9	0	0	9	0	0	0

lococci exhibited two different forms of methicillin resistance as shown in Fig 1 The first form (type 1) was similar to the coagulase—positive staphylococci The second form (type 2) was characterized by a distinct MIC value with growth at 12 5 but not at all at 25 µg/ml methicillin Bacteria with this methicillin resistance pattern were sensitive to all common antibiotics

Production of Enterotoxin B and other Factors

As demonstrated in Fig 2, 66 out of 80 methicillin resistant coagulase—positive staphylococci (82 5 per cent) were capable of producing enterotoxin B Among the coagulase—negative ones neither type 1 nor type 2 strains produced enterotoxin B Beta—haemolysin was produced by 53 of the coagulase—positives but none of the coagulase—negatives 9 of the *S aureus* (11 per cent) were lipase positive This is in contrast with the coagulase—negative variants which were all lipase positive

Elimination of DNase Activity

Altogether about 9000 colonies were tested for loss of DNase after treatment of 4 *S aureus strains (1779, 4516, 4916, 6969)* with EMS, MNG, Etbr or uv, which is to be compared with about 6000 untreated control colonies (Fig 3)

Only colonies growing on methicillin plates were further studied From these colonies examined only 2 were accepted as DNase negative mutants These two strains were designated A 114 and E 29 They were derived from DU 4916 by EMS and MNG treatment, respectively

Some other DNase-negative strains were found, but in all these the antibiogram was not the same as that of the parent strain and were either untypable or had different phage types For this reason their mutant status was considered not proved and were therefore discarded

Characterization of Mutants

The properties of A 114 and E 29 were further investigated and Fig 4 shows their

TABLE 3 *Number of Methicillin—Resistant Colonies Checked for DNase Production on DN Agar Plates after Treatment of Four Strains of* S aureus *with EMS, MNG Etbr and uv DNase—Positive = nuc, DNase—Negative = nuc⁻*

Strain	Number of colonies on DN agar									
	EMS		MNG		Etbr		uv		untreated	
	nuc⁺	nuc	nuc⁺	nuc	nuc⁺	nuc	nuc⁺	nuc	nuc⁺	nuc
4916	1712	1	331	1	1430	0	900	0	3100	0
6969			1500	0			1200	0	900	0
4516	50	0	159	0			200	0	1250	0
1779			679	0			1009	0	682	0
	1762	1	2669	1	1430	0	3309	0	5932	0

16*

TABLE 4 *Phenotypic Patterns of* S aureus *DU 4916 and Two nuc Mutants Derived from It*

Strain	4916	A 114	E 29
Phage type	NT	NT	NT
(RTD × 1000)	29/47/54/77/88	29/79/47/54/77/88	47/53/54/77/88
Antibiotics PcG	R	R	R
Eryt	S	S	S
Strep	R	R	R
Tetr	R	R	R
Chlor	S	S	S
Met 30°	R	R	R
DNase	+	—	—
Coagulase	+	+	—
α-Haemolysin	1/128	1/64	1/16
β-Haemolysin + 37°	1/16	1/4	1/2
β-Haemolysin + 4°	1/32	1/16	1/4
δ-Haemolysin	1/64	1/16	1/32
Enterotoxin B	+	+	+
Lipase	—	—	—
Phosphatase	+	+	—
Anaerobic Mannitol	+	+	—

characteristics in comparison with DU 4916 Mutant A 114 was still coagulase and enterotoxin B positive The only marker lost was DNase. Mutant E 29 on the other hand was negative for coagulase, phosphatase and anaerobic mannitol as well but still capable of producing enterotoxin B

DISCUSSION

Coagulase—negative staphylococci examined in this work exhibited two different forms of methicillin resistance One form was heterogeneous and highly resistant, similar to that described for *S aureus* (typ 1), the other form was a low resistance homogeneous type (*type 2*)

In the methicillin resistant *S aureus* strains, all of which exhibited the highly resistant heterogeneous form, there was a correlation between methicillin resistance and enterotoxin B production This was not observed in the coagulase-negative staphylococci none of which produced enterotoxin B

There is little information in the literature on enterotoxin producing *S albus* (8) However, our nuclease—negative mutants from enterotoxin—producing, methicillin—resistant S aureus were not similar to the wild type coagulase-negative strains in that they retained enterotoxin B production These results do not indicate a linkage between coagulase, nuclease and enterotoxin B production

The low number of detected coagulase—and nuclease—negative colonies (c 0 03 per cent) suggests that the genes responsible for these functions are not located extra-chromosomally The total rate of loss is not known since mutants were selected on methicillin containing agar *Dobrzanski et al* (10) reported a Co⁺Nuc mutant (similar to our mutant A 114) after treatment with MNG and this corresponded to a frequency of c 0 01 per cent *Omenn et al* (16) obtained 0 1 *per cent* Co Nuc mutants also with MNG, but the frequency of Co⁺Nuc *mutants* was 0 02 *per cent*

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THE EFFECT OF METABOLIC INHIBITORS ON THE PRODUCTION OF VACCINIA HAEMAGGLUTININ

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When synthesis of vaccinia DNA was inhibited no haemagglutinin (HA) was produced The antiviral compound isatin β thiosemicarbazone (IBT) also arrested the formation of vaccinia HA It is therefore concluded that the synthesis of vaccinia HA depends on late protein synthesis We have confirmed earlier findings that 5 bromodeoxyuridine (BUdR) does not inhibit HA production in doses which completely arrest production of infectious virus In contrast to earlier findings, mitomycin C inhibited HA production in our experiments The new antiviral compound rifampicin had no or little effect upon vaccinia HA production

Burnet & Stone (3) showed in 1946 that vaccinia virus-infected cells produce a haemagglutinin (HA) which is not part of the virion The HA is a pleomorphic particle with a diameter of about 50 mμ (14)

Recombination studies suggest that the information for the formation of the haemagglutinin resides in the viral genome (5 16)

Many studies have been made of the effect of metabolic inhibitors on the biosynthesis of poxviruses (cf review *Joklik, 1966*), but few reports deal with HA production

Mitomycin C was found by *Oda* (15) to allow the synthesis of HA the production of infectious virus being completely arrested *Loh & Payne* (11) found that 5 fluorodeoxyuridine (FUdR) markedly decreased the yield of vaccinia HA (cf also (19)) In a recent study *Baxby & Rondle* (2) examined the effect of several inhibitors on the production of vaccinia and cowpox HA They confirmed the findings by *Oda* (15) that mitomycin C without affecting the synthesis of HA arrested the production of infectious virus and found that this applied also to 5 bromodeoxyuridine On the basis of this they suggested that inoculum virus codes for the production of HA

The purpose of this study has been to define more accurately the stage in the replication cycle at which the synthesis of HA takes place We have reinvestigated the effect of several different metabolic inhibitors, and included others among them the new antiviral compound rifampicin which has been studied in more detail

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MATERIAL AND METHODS

Virus strain The vaccinia strain used was Statens Seruminstitut smallpox vaccine strain (Statens Seruminstitut Copenhagen) obtained from Dr *A Harboe* Oslo

Cells and media The 9809 subline of HeLa cells and the RK 13 cell line were obtained from Orion OY, Helsinki The cell monolayers were routinely cultured at 36° C in a humidified atmosphere containing 5 per cent CO_2 and passaged twice a week Growth medium consisted of Eagle's minimal essential medium (MEM Gibco Grand Island, N Y), supplemented with 10 per cent serum from newborn calf (Orion) The cells were detached from the glass by washing with GKN (0 137 M NaCl and 0 054 M KCl in a 1 mg/ml glucose solution) and were subsequently treated with 0 25 per cent trypsin (Difco 1 250) in GKN

Vaccinia antiserum The vaccinia antiserum was produced in rabbits by scarification of the skin and later by intravenous injections with the calf lymph smallpox vaccine

Inhibitors A commercial medicinal preparation of actinomycin D (Lyovac Cosmegen 2 5 per cent (W/v) actinomycin D in mannitol Merck Sharp & Dohme, Rahway, N J) was used

Mitomycin C (Sigma Chemical Co, St Louis Mo) was kept in the dark in the original ampoules and all experiments with this compound were performed in the dark or in subdued light

5 Fluorodeoxyuridine (FUdR) was a gift from Hoffmann La Roche Basel Switzerland. 5 Iododeoxyuridine (IUdR) 5 bromodeoxyuridine (BUdR) and cytosine arabinoside (ara C) were purchased from Calbiochem Los Angeles Calif, cycloheximide and hydroxyurea from Sigma Chemical Co St. Louis Mo

Rifampicin (CIBA Basel was dissolved in a small volume of medium made alkaline by NaOH and then diluted with culture medium to the desired concentration

Ter ... ift from ... dissolved ... diluted

... curves High titre virus stocks were prepared by subjecting 48 hr infected HeLa cells to three cycles of freezing and thawing and subsequent treatment for 5 min at 15 KC in an MSE Mullard ultrasonic disintegrator Cell debris was centrifuged off in a 10 min spin at 1 000 *g* and virus was pelleted by centrifugation at 27 000 *g* in a Servall Superspeed RC 2 refrigerated centrifuge The virus pellet was dissolved in a small volume of Hanks balanced salt solution (BSS) *Growth experiments* were performed with monolayers of HeLa cells in tubes (16 x 125 mm Bellco Vineland N J) A multiplicity of infection of ≈ 10 plaque forming units (p f u) per cell was employed to obtain a single-step growth curve The virus inoculate was removed after absorption of virus for 1 hr at 36° C and 1 ml of medium containing 10 per cent serum from newborn calf and the respective inhibitors was added At various intervals after infection the growth was stopped by placing the tubes in a —70° C ice chest Thereafter the cells were frozen and thawed three times and the cell debris centrifuged off at 2,000 *g* for 10 min The supernatant was used for infectivity titrations

The experiments using two successive inhibitors were performed in HeLa monolayers in 16 × 125 mm tubes. Before adding the second inhibitor the cultures were washed three times with Hanks BSS Otherwise the experiments were conducted as above

Virus infectivity titrations These were performed with monolayers of HeLa cells in 50 mm plastic Petri dishes (Nunc) by the plaque assay technique described by *Lindemann & Gifford* (10) Ten-fold serial dilutions of the virus were made in Eagle's MEM without serum and appropriate dilutions were inoculated onto the plates in 0 2 ml volumes After adsorption for 1 hr at 36° C, the plates were washed with Hanks BSS to remove unadsorbed virus and Eagle's MEM containing 2 per cent serum from newborn calf was added. Visible plaques were counted after incubation for 48 hr Titres were expressed in p f u /ml

HA and haemagglutination inhibition (HI) titration HA and HI titrations were conducted essentially as described by *Kempe & St Vincent* (9) Titres were given as the reciprocals of the dilutions

RESULTS

The effect of several DNA, RNA, protein and vaccinia virus inhibitors on the production of vaccinia HA and infectious virus in HeLa cells is shown in Table 1 Among the DNA inhibitors, FUdR, mitomycin C hydroxyurea and ara C which inhibit the synthesis of viral DNA all inhibited completely the synthesis of both HA and infectious virus

In contrast to this BUdR and IUdR allowed the synthesis of considerable amounts of HA the synthesis of infectious virus being arrested The HA produced in BUdR and IUdR treated cells was shown to be virus specific, being inhibited by an anti vaccinia hyperimmune serum and not by the respective preimmune serum

Actinomycin D and IBT inhibited both HA and virus production completely

Rifampicin has been shown to stop the replication of vaccinia virus without markedly inhibiting vaccinia DNA synthesis (12) It

TABLE 1 *The Effect of Various Inhibitors on the Synthesis of Vaccinia Virus and HA*

Inhibitor		Virus titre at 32 hr (p f u /ml)	Inhibition (per cent)	HA titre at 32 hr
None		$5 6\times10^{7}$		64
BUdR	100 μg/ml	$9 0\times10^{4}$	99 8	16
IUdR	100 μg/ml	$5 8\times10^{4}$	99 9	8
FUdR	100 μg/ml	$1 0\times10^{5}$	99 8	1
Ara C	30 μg/ml	$1 6\times10^{5}$	99 7	<1
Hydroxyurea	5 mg/ml	$1 2\times10^{5}$	99 8	<1
Mitomycin C	10 μg/ml	$1 0\times10^{5}$	99 8	<1
Actinomycin C	0 5 μg/ml	$4 3\times10^{4}$	99 9	<1
IBT	20 μg/ml	$6 5\times10^{5}$	98 8	1
Cycloheximide	10 μg/ml	$1 8\times10^{5}$	99 7	<1
Rifampicin	200 μg/ml	$1 7\times10^{5}$	99 7	16
Rifampicin	100 μg/ml	$3 4\times10^{5}$	99 4	16
Rifampicin	20 μg/ml	$9 4\times10^{6}$	83 2	32

TABLE 2 *The Effect of Actinomycin D, Cycloheximide and IBT on HA and Virus Production after Treatment with 100 μg/ml Rifampicin for 8 hr*

Inhibitor after rifampicin treatment		Virus titre 12 hr after change of inhibitor (p f u /ml)	Inhibition (per cent)	HA titre
None		$2 0\times10^{6}$		16
IBT	20 μg/ml	$5 0\times10^{4}$	97 5	<1
Actinomycin D	0 5 μg/ml	$3 7\times10^{5}$	81 5	1
Cycloheximide	300 μg/ml	$8 7\times10^{4}$	95 6	<1
Rifampicin	100 μg/ml	$7 9\times10^{4}$	96 1	8

has recently been shown that rifampicin prevents the formation of a vaccinia core polypeptide from a precursor molecule (22) In our experiments 100 μg/ml rifampicin arrested the synthesis of infectious virus, whereas HA was produced to titres which after 32 hrs were lower than in the controls, although within the limit of the experimental error The HA was shown to be virus specific when examined by the HI test

Sequential Inhibition with Rifampicin and other Inhibitors

The inhibitory effect of rifampicin can readily by reversed by washing out the antibiotic (8 12) In this way it is possible to examine the effect of other inhibitors on the same and later stages in the replication cycle

Vaccinia-infected cells were incubated with 100 μg/ml of rifampicin for 8 hr After washing three times with Hanks BSS 300 μg/ml cycloheximide 0 5 μg/ml of actinomycin D, and 20 μg/ml of IBT were added to the respective cultures

As can be seen from Table 2 there was only slight production of HA in the presence of actinomycin D No HA was produced when cycloheximide or IBT was added Some infectious virus was produced in the presence of actinomycin D while cycloheximide and IBT allowed no production of virus

DISCUSSION

The formation of HA and infectious virus was inhibited by FUdR, ara C, hydroxyurea and mitomycin C, which all inhibit the synthesis of DNA without being incorporated into it It is thus clear that synthesis of viral DNA is a prerequisite for the production of HA

IBT also inhibited the production of HA and infectious virus. This compound interferes with the function of late vaccinia mRNA without affecting earlier events in the replication cycle (23). HA synthesis accordingly depends on late viral protein synthesis.

Our results with mitomycin C contrasted with those reported by *Oda* (15) and later by *Baxby* & *Randle* (2). These investigators obtained HA production in the absence of infectious virus with this inhibitor, which led to the assumption that HA production represented an early event in the replication cycle. Mitomycin C is considered to cause the breakdown of newly formed DNA (21), but varying effects of this antibiotic have been described. Inhibition of *E. coli* has been obtained under conditions where DNA breakdown *did* not occur (20), and evidence has been presented that in the presence of mitomycin C the two strands of DNA can be linked *together* so that no replication takes place although the DNA may still function as a *primer for* RNA (16). Mitomycin C is very unstable, and we have found that different batches vary considerably in their effect. Identical results were obtained with the RK 13 cell line used by *Baxby* & *Randle* (2) (not included under *Results*).

In accordance with earlier findings (2) we obtained HA production in the presence of BUdR and IUdR, thymidine analogues which are incorporated *into* DNA. Viral antigens have also been found to be produced in BUdR treated cells (1). *Joklik* (7) warns against the drawing of conclusions about early and late *synthesis of* vaccinia proteins from the effect of these inhibitors. The degree of incorporation varies, and it is difficult to foresee whether the resultant DNA in BUdR or IUdR inhibited cells will be a good template, and whether the information in the mRNA produced will yield a functional protein. The production of HA in the presence of these inhibitors can accordingly not be taken as direct *evidence* against the view presented here that DNA synthesis is a requisite for DNA production.

The finding that virus inhibiting doses of rifampicin have no effect on HA production is of considerable interest. It indicates that although the synthesis of HA depends on a late event in the infectious cycle, the formation of infectious virus is not required. Electron microscopy has shown that immature membranes are formed in vaccinia infected cells in the presence of rifampicin. The production of immature forms and infective virus does not however commence until the rifampicin is removed (6, 17). It is interesting to note that immature virus membranes can also be discerned in BUdR treated vaccinia infected cells, where HA but not infectious virus is produced (4). Their morphology corresponds rather well to that seen in rifampicin treated cells (6, 13, 17).

The inhibitory action of cycloheximide, actinomycin D and IBT on the synthesis of HA and virus after the removal of rifampicin shows the requirement of *de novo* RNA and protein synthesis for the continued production of HA and for completion of the infectious cycle. The lifetime of the mRNA concerned is not known, so the possibility cannot be excluded that the need for additional mRNA and protein synthesis is due *to* a rapid inactivation of late mRNA.

Nagayama et al. (13) and *Pennington et al.* (17) also failed to show virus production in the presence of protein inhibitors after removal of the rifampicin. *Moss et al.* (12) on the other hand obtained virus growth in the presence of cycloheximide in a similar experiment. The reason for this discrepancy is difficult to explain.

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HETEROGENEITY IN THE REACTIVITY OF ANTIBODIES WITH KAOLIN

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The reactivity of kaolin with different antibodies has been examined. Several antibodies against bacterial, protozoal and viral agents, as well as cold agglutinins, were not removed by kaolin treatment of sera, while mononucleosis antibodies, isoagglutinins, human antibodies to rabbit red cells and rheumatoid factor were almost completely removed by this treatment. The difference in reactivity among these antibodies must be due to an unknown heterogeneity in their physico-chemical properties.

Kaolin, a water-containing aluminium silicate, is widely used for removing the non-specific serum inhibitors of viral haemagglutinins. The inhibitors concerned are β-lipoproteins, which inhibit rubella and arbovirus haemagglutination, and sialoglycoproteins, which inhibit myxo- and paramyxovirus haemagglutination. Some investigators claim that kaolin also removes antibodies, particularly those of the IgM class (2, 3, 4, 10, 11), while other investigators have not been able to confirm this (1, 7, 9, 15, 20). *Schmidt & Lennette* (15) point out the importance of carrying out the kaolin absorption procedure at pH 9.0 to avoid or minimize removal of antibodies. *Cabasso et al.* (1) showed that rubella antibodies can be removed from an immune globulin preparation, but not from serum. They presented evidence that antibodies in serum are protected by albumin against adsorption to kaolin.

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The above mentioned results were obtained from quantitative determinations of classes of immunoglobulins or from comparison of antibody titres after different pre-treatments for removal of inhibitors. In the first case, nothing is known about the specificity of the antibodies removed. In the second case, the conclusions regarding the effect of kaolin are based on the assumption that the other pre-treatments do not remove antibodies or leave inhibitors.

Our original intention was to see if the kaolin method could be relied upon for removal of rubella haemagglutinin inhibitors. Some of these results have been published earlier (6). In the course of these investigations we observed a striking variation in the effect of kaolin on antibodies of different specificities. This property of kaolin has been examined further in the present study.

MATERIAL AND METHODS

Sera. The sera were selected from specimens sent to the laboratory for routine testing, from vaccinated students, from members of the laboratory staff and from immunized rabbits. A pool of normal human

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The reactivity of kaolin with different antibodies has been examined Several antibodies against bacterial protozoal and viral agents as well as cold agglutinins were not removed by kaolin treatment of sera while mononucleosis antibodies, isoagglutinins, human antibodies to rabbit red cells and rheumatoid factor were almost completely removed by this treatment The difference in reactivity among these antibodies must be due to an unknown heterogeneity in their physico-chemical properties

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while other investigators have not been able to confirm this (1 7, 9 15, 20) *Schmidt* & *Lennette* (15) point out the importance of carrying out the kaolin absorption procedure at pH 9 0 to avoid or minimize removal of antibodies *Cabasso et al* (1) showed that rubella antibodies can be removed from an immune globulin preparation but not from serum. They presented evidence that antibodies in serum are protected by albumin against adsorption to kaolin

The above mentioned results were obtained from

removal of inhibitors In the first case, nothing is known about the specificity of the antibodies removed In the second case, the conclusions regarding the effect of kaolin are based on the assumption that the other pretreatments do not remove antibodies or leave inhibitors

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MATERIAL AND METHODS

Sera The se
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sera was also included All sera were kept at —20° C and were heat inactivated before use

Treatment of sera The sera were treated as described for removal of rubella virus haemagglutinin inhibitors with *kaolin* (5) with *heparin manganese chloride* (2), and with *dextran sulphate* (6) Treatment with *Mercaptoethanol* (ME) was performed by incubating equal volumes of serum and 0 2 M ME at 37°C for 30 min

Serological tests The antistreptolysin (AST), antistaphylolysin (ASTA), Widal Paul & Bunnell Waaler Rose, Wassermann (WR), Gonococcus (Gc) complement fixation (CF), and cold agglutination tests were carried out in the routine laboratory by the standard techniques Antibodies to *Toxoplasma gondii* were titrated by the indirect haemagglutination (IHA) test (8) Isoagglutinins and antibodies to rabbit red cells were titrated by mixing serum dilutions and a one per cent suspension of red cells 0 25 ml of each Rubella virus haemagglutination inhibitors were assayed by the method of *Stewart et al* (16) Serum agglutinins to *Staphylococcus aureus* strain Cowan I were titrated by agglutination on slides (13) and antibodies to polysaccharide A of *Staph aureus* were titrated by the IHA method (14)

All dilutions started at 1 in 10 which is the final dilution after kaolin treatment

All direct haemagglutination tests were read after centrifugation for 60 sec in an Adam s serofuge

RESULTS

Treatment of Some Sera with Kaolin, Heparin $MnCl_2$ and Dextran Sulphate

Heparin $MnCl_2$ and dextran sulphate had no effect upon the antibody titres (Table 1) while kaolin removed almost all antibody in some systems (*e g* mononucleosis antibodies rheumatoid factor anti rabbit red cells), but had no effect on the titres in other systems (*e g* AST ASTA, cold agglutinins) Kaolin accordingly differed from heparin MnCl and dextran sulphate in that it removed some antibodies

The Effect of Kaolin Studied with more Sera

To obtain more information about the consistency of this property of kaolin the study was extended to more sera and other antigen antibody systems The results are summarized in Table 2, which needs a few comments concerning variations among individual sera and possible classification of immunoglobulins

AST The titres were the same before and after treatment with kaolin except in one serum, in which a 1 step titre reduction was found ME treatment caused no reduction in titre

Widal The sera examined were from individuals recently vaccinated with a *Salmonella typhi, S paratyphi A* and *S paratyphi B vaccine*, from a patient with a salmonellosis, from a pool of human sera and from an immunized rabbit All sera were examined for agglutinins against the respective O and H antigens The serum pool contained antibodies to *S typhi H* and *S paratyphi BH* which were resistant to treatment with ME Otherwise all reactivity was lost after treatment of sera with ME

The average titre reduction after kaolin treatment was 1 5 fold, expressed as indicated in the table The individual titres were either the same or one step lower except in one instance when a 4 fold reduction was observed In no case was a higher titre obtained Apparently kaolin removes some anti salmonella antibody

WR The average reduction in titre was 1 2 fold or slightly higher as two sera gave titres below 10 after kaolin treatment Of the 8 sera examined 5 had the same titre one serum showed a 1 step higher titre, two sera a 1 step reduction and one serum a 2 step titre reduction

Gc CF antibodies The two sera examined showed the same titre before and after treatment

Toxoplasma antibodies The results obtained were almost the same as for WR antibodies both with regard to average reduction and individual variation

Cold agglutinins The average reduction was 1 1 fold Four of the sera gave the same titre one serum a 1 step rise and two sera a 1 step reduction

Mononucleosis antibodies A more than 20-fold reduction in titre was obtained with considerable variations among the individual sera ranging from 4 to ≥ 32

Antibodies to rabbit red cells The listed 11 6 fold reduction figure is obviously too

low, since the titres were reduced to below detectable limits in 13 of the sera.

One serum had dominating IgG antibodies and showed a 4 fold reduction in titre. The other sera contained mainly IgM. The titre reduction varied from 2 fold to more than 32 fold.

A B isoantibodies. In most of the sera a 4 fold reduction was obtained with individual variations from 2 to ≧ 16.

Rheumatoid factor. The titres in the Waaler Rose test were reduced by a factor of more than 16. Considerable variations among the individual sera were also observed here,

TABLE 1 *Kaolin, Heparin-MnCl$_2$ and Dextran Sulphate Treatment of Sera. Antibody Titre in Different Antigen-Antibody Systems*

Serum	Type of antibody	Untreated	Treated with		
			kaolin	heparin MnCl	dextran sulphate
4940	Mononucleosis antibodies	640	< 10	320	320
4938	Mononucleosis antibodies	320	< 10	160	160
4941	Mononucleosis antibodies	1 280	< 10	640	1 280
4967	Mononucleosis antibodies	320	< 10	320	160
6502	Cold agglutinins	640	320	320	640
6500	Cold agglutinins	320	160	160	320
44147	Rheumatoid factor	160	< 10	160	—
44537	Rheumatoid factor	160	< 10	80	40
47262	Rheumatoid factor	80	< 10	40	40
47122	Rheumatoid factor	20	< 10	20	40
E S	IgM human anti rabbit red cells	1 280	10	320	1 280
W 46	Antibodies to polys A	10 240	10 240	10 240	
51034	AST	120	100	100	[illegible]
49758	AST	100	100	100	100
51023	ASTA	6	5.5	6	4.5
51095	ASTA	5	5	5	5

— not examined

TABLE 2 *The Effect of Kaolin Treatment on Antibody Titres*

Number of sera	Antibody or serological test	Geometric mean titre		Reduction factor untreated : treated
		untreated	kaolin treated	
11	AST	871	813	1.1
7	Cold agglutinins	1 035	940	1.1
8	WR	26	21*	1.2*
16	Widal	134	88	1.5
10	*Toxoplasma g.* (IHA)	70	43	1.6
5	Agglut. *Staph. aureus*	211	106	2.0
4	Gonococcus CF	28	28	1.0
19	Mononucleosis	462	21*	22.0*
13	Waaler Rose	338	22*	15.4*
30	Human anti rabbit r.b.c.	167	14*	11.9*
11	Isoagglutinins (α β)	66	15*	4.4*

*Titre below detectable level in some sera after kaolin treatment. Geometric mean titre therefore possibly lower and the respective reduction factor values higher.

TABLE 3 *The Effect of the Amount of Kaolin on Antibody Titres*

Serum	Antibody	Untreated	Treated with kaolin		
			12 5 %	25 %	50 %
S R.	Anti rabbit r b c	2,560	160	160	160
P H S	Anti rabbit r b c	320	20	20	40
8368	Mononucleosis	2,560	640	160	40
R O	Mononucleosis	2,560	640	160	40
G F	Rheumatoid factor	5,120	320	160	160
1020	Rheumatoid factor	1,280	80	40	40
A D	Rubella HI		80	[illegible]	40
G F	Rubella HI		80	40	40

from a 2-fold to above 32-fold reduction The low reduction was obtained particularly with low-titred sera

The Effect of the Amount of Kaolin and Time of Incubation

The final concentration of kaolin is 12 5 per cent in the 1 in 10 dilution of serum The concentration was increased to 25 and 50 per cent, the effect on antibody titres in several systems being *shown in* Table 3 *It appears* that with the sera examined increasing amounts of kaolin did not result in a significant reduction of titres of antibodies to rabbit red cells, rheumatoid factor and rubella HI antibodies, while the amount of mononucleosis antibodies was further reduced

A 2-step absorption was also attempted (not included in the Table) After one absorption the titre of a mononucleosis serum was reduced from 2560 to 160 A second treatment with 12 5 per cent kaolin resulted in a titre below 10

Rubella HI inhibitor, mononucleosis antibody and cold agglutinin titres were examined after various times of incubation Rubella HI inhibitors were removed almost immediately while the mononucleosis antibodies had to be treated from 10 to 40 minutes for maximal absorption Prolonged treatment up to 18 hrs did not result in further absorption of antibody The cold agglutinin titre remained unchanged throughout the period

Elution of Antibody from Kaolin

Several procedures were tried for the release of the antibody bound to kaolin After being centrifuged off from the serum kaolin was suspended in buffers of different pH and ionic strength and incubated at 37° C or 56° C Increase of temperature and particularly of pH proved successful Mononucleosis and anti rabbit red cell antibodies were eluted at pH 11 0 at 37° C and at pH 9 0 at 56° C Rheumatoid *factor* was eluted at pH 11 0 at 37° C and at 56° C No elution occurred at lower pH values

DISCUSSION

From the degree of antibody titre reduction two groups of antibodies can be *distinguished*

1 Antibody titres reduced by one step or less
Rubella antibodies (cf (6))
Antistreptolysins
Antistaphylolysins
WR-antibodies
Gc-antibodies
Toxoplasma gondii antibodies
Normal agglutinins against *Staph aureus* strain Cowan I
Rabbit antibodies against polysaccharide A
Agglutinins to *Salmonella typhi*, *S paratyphi* A and B
Cold agglutinins

2 Antibody titres reduced, on an average, more than 4 fold

Mononucleosis antibodies

Antibodies to rabbit erythrocytes of IgM class

Isoagglutinins A and B

Rheumatoid factor

Most of the anti salmonella agglutinins in group 1 were ME sensitive and possibly be long to the IgM class. The cold agglutinins are known to be IgM. The other antibodies examined were ME resistant. Rubella IgM antibody titres have earlier been claimed to be not significantly affected by kaolin treatment (6 20). The lack of binding to kaolin is accordingly not limited to one class of immunoglobulins. Group 2 comprises antibodies known to belong wholely or mainly to the IgM class. One striking difference between the two groups is that group 1 consists, with the exception of the cold agglutinins of antibodies to bacterial and viral agents whereas group 2 consists of heterophile antibodies, isoagglutinins and autoantibodies.

It was considered of particular importance to establish that what was removed by kaolin was actually antibodies, and not one of the additional factors sometimes necessary for a serological reaction, e g complement, or the agglutinator described by *Tonder & Milgrom* (19). This was excluded since recomplementation of sera and addition of agglutinator containing serum did not restore the serological reactivity (not included under Results) and since antibodies were recovered from kaolin by raising pH and temperature. The present findings leave no doubt that some antibodies are strongly bound to kaolin. For the rheumatoid factor one possible mechanism of action is that the factor reacts with IgG bound to kaolin. *Heinblad* (21) has used kaolin in the same way as latex for demonstration of rheumatoid factor but he is reluctant to accept the common explanation that IgG-coated kaolin is agglutinated by the rheumatoid factor. He suggests that IgM-IgG complexes alter the milieu and thereby cause the kaolin particles to aggregate. *Tönder* (17) has shown that bentonite, another aluminium silicate, strongly binds rheumatoid factor. In contrast to our findings, rheumatoid factor could be eluted from bentonite only at a pH below neutrality.

In most cases residual titres were found in group 2, and an increase in the amount of kaolin had no effect except in the case of mononucleosis antibodies. This possibly reflects some heterogeneity among the antibodies concerned, similar to that known to exist for the rheumatoid factor (12) and the antibodies to rabbit erythrocytes (18), but this was not examined further.

The different reactivity of group 1 and 2 antibodies must be due to a hitherto unknown difference in physico-chemical properties between the two groups. It is an open question whether a distinction between anti-

Immunologists are inclined to deny such a distinction or at least a distinction between so-called immune and natural antibodies. Investigations are in progress to prepare a kaolin material for column chromatography and thereby study the elution patterns of different antibodies.

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ANTIGENIC STUDIES OF GENUS *MICROCOCCUS*

1. *Agglutination*

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Forty strains of micrococci representing all the 8 biochemical types of *Baird-Parker* (4) were examined for their content of bacterial agglutinogens and antigens sensitizing normal and tanned sheep erythrocytes. Special attention was paid to the urinary pathogens of type 3. Agglutination of the strains in 10 rabbit immune sera against all biochemical types before and after absorption showed that the *Micrococcus* strains have a number of agglutinogens without any systematic pattern. The majority of the agglutinogens are shared by many strains irrespective of biochemical type. Agglutinogens characteristic of the biochemical types were not demonstrated. Strains of the same biochemical type were, as a rule, not more related antigenically than strains of different types. The indirect haemagglutinations revealed that the micrococci have several shared antigens sensitizing normal and tanned sheep erythrocytes to agglutination in *Micrococcus* and *Staphylococcus aureus* immune sera.

The characterization of genus *Micrococcus* by its inability to attack glucose anaerobically (22) and its high per cent GC content (6, 2) has given a more reliable basis for the separation of its members from the staphylococci and other Gram positive cocci. The subdivision of genus *Micrococcus* into species or groups is however more questionable. *Evans* (8) and *Bohacek et al.* (6) are of the opinion that at present only two species, i.e. *M. luteus* and *M. roseus* should be recognized. *Baird-Parker* (4) separates micrococci into 8 types according to certain biochemical properties.

Knowledge of the antigens of micrococci may give information which can be expected to contribute to their taxonomy and classification. The recent recognition that micrococci of type 3 are capable of causing urinary infections (20, 13, 21) makes serologic investigations particularly desirable. Wall teichoic acids have been isolated from strains of micrococci by *Davison & Baddiley* (7) and *Baddiley et al.* (3) and three groups of strains have been distinguished. Serologic investigations of polysaccharides and teichoic acids from micrococci have been reported by *Losnegard & Oeding* (12) and *Oeding et al.* (18).

In an earlier investigation (*Oeding* (16)) of 18 strains classified as *M. luteus*, *M. roseus*, *M. conglomeratus*, and *M. varians* weak sharing of agglutinogens with staphylococci was reported. No serologic correlation to the above species of micrococci was registered, and the *Micrococcus* agglutinogens offered no basis for a type differentiation.

The intention of the present investigation has been to examine serologically representa-

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tives of the 8 *Baird-Parker Micrococcus* biotypes (4), particular attention being paid to type 3 This report will consider the antigens participating in the agglutination of bacteria and sensitized erythrocytes, whereas the precipitinogens will be treated in a subsequent paper

MATERIAL AND METHODS

Strains A total of 56 strains classified as belonging to genus *Micrococcus* was included in the total serologic investigation They were Gram positive cocci of varying size producing catalase but not coagulase and not producing acid from glucose anaerobically (22) The origin of the strains was

Professor *A T Pereira*, Instituto Bacteriológico Câmara Pestana, Lisboa, Portugal 6 strains isolated from urinary infections

Dr *A P Roberts*, Queen Charlotte s Hospital, London, England 5 strains isolated from urinary infections, type 3

Dr *R G Mitchell*, The Churchill Hospital, Oxford, England 10 strains isolated from urinary infections, type 3

Dr *M S Pohja*, Hameenlinna, Finland Strain 234, isolated from meat

Dr *A C Baird Parker*, Unilever Research, Bedford, England 2 type 4 strains, 3 type 6 strains 3 type 8 strains, isolated from meat or dairy products, or origin unknown

Losnegard & Oeding (12) 11 strains isolated from urine, 1 from blood, 1 from pus

Diagnostic laboratory of the Institute 10 strains isolated from urine 1 from blood 2 from pus

Staphylococcus aureus strain Cowan I was included in the haemagglutination experiments

The biochemical typing of the strains according to *Baird Parker* (4) presented certain problems with regard to the determination of glucose oxidation and the production of acetoin As the plate method seemed to give more reliable results than in tube, oxidation of glucose was determined according to the results with the former method Three methods for determination of acetoin production all gave somewhat different results The method adopted which gave results consistent with those of other authors from whom typed strains had been received was inoculation in tubes containing glucose phosphate peptone water (5, 11) incubation at 37° C for 2–4 days and addition of O Meara s reagent (19)

According to the biochemical examinations 7 strains belonged to type 1, 6 to type 2 31 to type 3, 2 to type 4 1 to type 5, 3 to type 6 3 to type 7, and 3 to type 8 All the strains belonging to type 1, 2 3 and 5, and two of the type 7 strains were isolated from urine, blood or pus of patients Of the type 3 strains, 21 had caused urinary infection All type 4, 6, and 8 strains, and one of type 7, were isolated from meat or dairy products or their origin was not known

In the present report 17 type 3 strains and all the 25 strains belonging to the other types were used, except one type 6 strain and one type 8 strain, which agglutinated spontaneously

The sensitivity to novobiocin was determined by the agar difusion technique using paper disks with a concentration of 5 μg An inhibition zone corresponding to a concentration of novobiocin below 3 μg/ml was read as sensitive Thirty seven strains were resistant to novobiocin, among these all type 3 strains except 3 With the exception of one type 1 and 1 type 2 strain, all strains of types 1, 2 7, and 8 were sensitive The results are in accordance with earlier reports on novobiocin sensitivity (20 13, 14), with the exception that *Mitchell & Baird Parker* (14) found that all glucose-oxidizing types i e all except types 7 and 8, were resistant

Sera Immune sera were produced by intravenous injections of formalin killed bacteria (grown at 37° C) (15) into New Zealand white rabbits of the Institute s breed Samples were drawn before immunization for the examination of natural antibodies Immune sera were produced against 3 strains of type 3 and 1 strain of each of the other types (see Tables) The strains used for immunization are referred to below as the type strains Immune serum against *Staph aureus* strain Cowan I was used in the haemagglutination experiments

Agglutination Agglutination was carried out on slides using live bacteria grown at 37° C (15, 10)

Indirect haemagglutination Normal and tanned sheep erythrocytes were sensitized with phosphate-buffered saline extracts of the bacteria and tested for agglutination in dilutions of the immune sera (17) The reactions were incubated 30 min at 37° C and then overnight at room temperature and read 1 as a pattern, 2 after gentle shaking 3 15 min after vigorous shaking

RESULTS

Agglutination in Preimmune Sera

Preimmune serum from all the rabbits used for immunization was examined for the presence of natural agglutinins Each undiluted preimmune serum agglutinated from 2 to 10 strains and only scattered reactions were observed with serum diluted 1 4, above which no strain agglutinated The natural agglutinins showed no prevalence for any biochemical type Natural antibodies, therefore, presented no problem for the evaluation of immune agglutinins

TABLE 1 *Agglutination of "Type Strains" in Micrococcus Immune Sera*

	Immune sera against strain									
Strains	4036 (1)	4275 (2)	s/m 26 (3)	Bri U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
4036	*4096*	1024	16	2048	1024	32	16	16	64	8
4275	64	*512*	32	32	32	8	1	8	32	2
s/m 26	64	16	*256*	8	16	32	2	16	8	4
Bri U99	32	16	32	*64*	16	64	8	32	16	16
9145	64	16	16	8	*1024*	32	–	4	8	–
1849	32	8	16	8	(8)	*256*	8	64	[illegible]	8
10653	(4)	4	2	1	8	1	*128*	2	–	–
1463	128	256	8	64	16	32	32	*2048*	64	32
5350	8	64	16	16	16	128	–	32	*2048*	8
1474	–	–	–	–	–	–	–	–	–	[illegible]

Numbers in brackets biochemical type
– no agglutination in undiluted serum
Titres in brackets preimmune titre one step lower
Titres given as reciprocal values

TABLE 2 *Agglutination of biochemical Type 1 strains in Micrococcus Immune Sera*

	Immune sera against strain									
Strains	4036 (1)	4275 (2)	s/m 26 (3)	Bri U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
4036	*4096*	1024	16	2048	1024	32	16	16	64	[illegible]
3973	32	*64*	*8*	*16*	2	8	1	8	4	2
3984	512	64	16	256	256	128	2	32	16	16
4150	*16*	*128*	32	16	8	8	–	8	*64*	2
5383	–	–	–	2	4	–	–	–	256	–
5878	64	*64*	*4*	*32*	4	2	2	4	2	1
9013	32	*64*	256	128	32	32	32	256	32	32

Numbers in brackets biochemical type
– no agglutination in undiluted serum
Titres given as reciprocal values

Agglutination in Immune Sera

The 10 *Micrococcus immune sera* were tested by agglutination against the corresponding "type strains" (Table 1). With the exception of strain 1474 *(type 8) all the* strains were agglutinated by nearly all the immune sera. *Three sera had rather low homologous* titres but one of them (Bri U99) agglutinated one heterologous strain quite strongly. The weak Bri U99/anti Bri U99 reaction may therefore be explained as a result of blocking antigens. The heterologous titres were usually low, 4 *to 7 steps below* the homologous titre, and were *only in a few* instances of a level comparable to the homologous titre. The results indicated that the strains share agglutinogens to a considerable degree, but also that rather strong specific agglutinogens are present. The 3 type 3 strains did not appear to be more closely related than the other strains or to behave differently from these in the agglutinin reaction.

The strains of each biochemical type were then tested in all immune sera. The 7 type 1 strains (Table 2), the 2 type 6 strains, the 3 type 7 strains, and the 2 type 8 strains did not agglutinate more strongly in the immune serum of the same type than in several other sera. The cross reactions were of the same

TABLE 3 *Agglutination of biochemical Type 2 Strains in Micrococcus Immune Sera*

	Immune sera against strain									
Strains	4036 (1)	4275 (2)	s/m 26 (3)	Bri U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
4275	64	*512*	32	32	32	[illegible]	1	8	32	2
3519	64	32	256	128	32	64	16	256	32	32
4044	32	256	64	16	16	8	[illegible]	[illegible]	64	–
4279	32	256	(4)	32	–	2	–	8	2	4
5596	256	512	8	1024	8	16	16	32	32	8
5855	32	256	(8)	64	–	16	8	32	8	8

Numbers in brackets biochemical type
– no agglutination in undiluted serum

Titres in brackets preimmune titre one step lower
Titres given as reciprocal values

level as seen in Table 1 for the 'type strains Thus the strains of each of these types were not more related to their respective "type strains' than to the 'type strains' of other types In contrast to this, 5 of the 6 type 2 strains agglutinated in practically the same dilution of the type 2 serum (Table 3), indicating that these strains were antigenically similar This was also true of the 2 type 4 strains, whereas the 17 strains belonging to type 3 gave more mixed reactions, indicating that some strains were antigenically similar whereas the majority were not more related to the type 3 serum strains than to other "type strains"

Absorption of Immune Sera

Each immune serum diluted 1 10, was absorbed with one strain usually the 'type strain , of all the 8 biochemical types Each of the absorbed sera was then tested by agglutination with all the type strains The titres were strongly reduced after absorption and were now usually not higher than 1 10 to 1 30 In sera which had been absorbed with heterologous 'type strains the antibody content against the homologous strains was reduced to the same level, but the sera were never exhausted Several strains agglutinated exclusively in the homologous serum after absorption with a heterologous type strain The type 1 strain 4036 was particularly effective in removing shared agglutinins from the sera

The absorption experiments confirmed that the "type strains" have shared agglutinogens, but, in contrast to the experiments with unabsorbed sera, indicated that shared agglutinogens constitute the bulk of the agglutinogens whereas the more specific agglutinogens are rather weak

The absorbed sera were then tested against all strains of that biochemical type to which the strain used for absorption belonged The results for the strains belonging to the biochemical types 1 and 2 are given in Tables 4 and 5 respectively Comparing these results with those before absorption presented in Tables 2 and 3, indicates that the type 1 respectively type 2, strain used for absorption has reduced the titres of the strains of their respective types to a considerable extent Three strains of each type have agglutinogens the antibodies to which the type strain has not been able to remove these agglutinogens being shared with a varying number of type strains belonging to other types Strain 9013 of type 1 shares such agglutinogens with all the type strains except that of type 1 and strain 3519 of type 2 shares them with the majority of the other 'type strains

Absorption of the immune sera with the three type 3 strains resulted in a considerable reduction of the titres against the 17 type 3 strains The results after absorption with strain s/m 26 are given in Table 6 It appears that this strain has removed the bulk of the antibodies of all sera agglutinating type 3 strains and that some sera have been exhausted Several type 3 strains however

TABLE 4 *Absorption of All Micrococcus Immune Sera with "Type Strain" 4036 of biochemical Type 1 Agglutination [illegible] Absorbed Sera with All Type 1 Strains*

	Absorbed immune sera								
Strains	4275 (2)	s/m 26 (3)	Bn U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
4036	–	–	–	–	–	–	–	–	–
3973	–	–	–	/	–	/	–	/	/
3984	–	–	–	–	–	/	–	–	–
4150	80	–	–	–	–	–	10	40	/
5383	/	/	/	/	/	/	/	80	/
5878	–	/	–	/	/	/	/	/	/
9013	10	40	40	10	10	10	40	10	10

Numbers in brackets biochemical type
– no agglutination in serum dilution 1 10
/ titre < [illegible] in unabsorbed serum
Titres given as reciprocal values

TABLE 5 *Absorption of All Micrococcus Immune Sera with "Type Strain" 4275 of Biochemical Type 2 Agglutination in Absorbed Sera with All Type 2 Strains*

	Absorbed immune sera								
Strains	4036 (1)	s/m 26 (3)	Bn U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
4275	–	–		–	–	–	–	–	–
3519	10	40	20	–	10	–	80	–	–
4044	–	–	–	–		/	–	–	/
4279	–	/	–	/	/	/	–	/	/
5596	10		10	–	–		–	–	–
5855	–	–	10	/	–	–	–	–	–

Numbers in brackets biochemical type
– no agglutination in serum dilution 1 10
/ titre < 8 in unabsorbed serum
Titres given as reciprocal values

have agglutinogens the antibodies to which have not been removed by strain s/m 26 Such antibodies are mainly found in the two type 3 sera but also in the type 1 and 2 sera This suggests that at least some type 3 strains are more related to each other than to the "type strains" of the other types

These results and additional absorptions of the three type 3 sera with a number of type 3 strains indicated that the "type strains" s/m 26 and Bn U99 and some other type 3 strains are rather similar Several type 3 strains, however appeared to be as closely related to strains of other types

Indirect Haemagglutination

Normal and tanned sheep erythrocytes were sensitized with extracts of the *Micrococcus* type strains In addition, tanned cells were sensitized with an extract of *Staph aureus* strain Cowan I The sensitized cells were agglutinated in dilutions of the 10 *Micrococcus* immune sera and *Staph aureus* serum Cowan I All strains except strain 1474 of type 8 had antigens which sensitized both normal and tanned sheep erythrocytes to agglutination in all the sera There was little difference in the homologous and heterologous titre values and the results indicated

TABLE 6 *Absorption of All Micrococcus Immune Sera with 'Type Strain' s/m 26 of Biochemical Type 3 Agglutination in Absorbed Sera with All Type 3 Strains*

	Absorbed immune sera								
Strains	4036 (1)	4275 (2)	Bri U99 (3)	9145 (3)	1839 (4)	10653 (5)	1463 (6)	5350 (7)	1474 (8)
s/m 26	–	–	–	–	–	–	–	–	–
s/m 9	10	–	15	15	–	/	–	/	
877	–	–	10	–	–	/	–	/	/
51/3	30	10	15	30	30	/	–	10	
CC	10	10	30	–	–	/	–	1	
Bri U14	–	/	/	/	/	/	/	/	/
Bri U99	–	10	10	–	–	–	–	–	–
16888	/	/	–	–	–	/	–	/	/
17039	/	/	–	–	–	/	–	/	–
G & T	/	/	/	/	/	/	/	/	/
C Munro	–	/	10	/	–	/	–	/	/
69555	/	10	/	/	/	/	/	/	/
1622	–	–	–	–	–	–	–	10	–
3826	30	10	10	15	–	/	–	–	/
8796	–	–	10	15	–	/	–	/	–
9145	10	–	–	60	–	/	/	–	/
9510	/	/	/	/	/	/	/	/	/

Numbers in brackets biochemical type
– no agglutination in serum dilution 1 10
/ titre < 8 in unabsorbed serum
Titres given as reciprocal values

that the strains contained several shared antigens sensitizing both normal and tanned cells There was also a strong sharing of antigens with the *Staph aureus* strain Cowan I Cells sensitized with strain 1474 of type 8 were agglutinated in few sera only, *e g* not in serum Cowan I, and then in low titres

DISCUSSION

No apparent correlation was demonstrated by *Oeding* (18) between the results of agglutination and the 4 species of *Micrococcus* examined, *viz M luteus, M roseus M conglomeratus*, and *M varians* Of these only the first two in the opinion of *Evans* (8) and *Bohaček et al* (6), deserve the rank of species It was concluded by *Oeding* that the uncertainty connected with the classification, and thereby the selection of the strains, might have obscured any correlation with serologic results In the present investigation a larger material of *Micrococcus* strains selected on the basis of the biochemical typing of *Baird Parker* (4), has been examined

Forty strains, none of which were found to produce acid from glucose anaerobically were examined by agglutination All the 8 biochemical types were represented, types 4–8, however, by only a small number of strains The largest number of strains was of type 3, this type being considered to be of particular interest because of its reported ability to cause urinary infections (20 13 21) Although the examination for glucose oxidation and acetoin production presented certain problems, the typing of the strains was considered reliable

Natural agglutinins against the strains were found only in small amounts in the rabbit sera before immunization and represented no problem for the evaluation of the immune reactions Slightly higher titres of natural antibodies against micrococci were reported by *Oeding* (16), who concluded that they were of several types

Agglutinations in the 10 unabsorbed sera and after absorption with representatives of all biochemical types clearly showed that the *Micrococcus* strains have a number of agglutinogens without any systematic pattern The majority of the agglutinogens are shared by many strains, irrespective of the biochemical type Although some agglutinogens have more limited distribution, the designation type or specific antigen is probably not justified The results indicate that the strains differ quantitatively rather than qualitatively in their content of agglutinogens A corresponding conclusion was drawn by *Oeding* (16)

Agglutinogens characteristic of the biochemical types could not be demonstrated On the whole the strains of one biochemical type were no more similar antigenically than strains of different types There were, however certain exceptions to this rule *e g* a close relationship between 5 of the 6 type 2 strains and between some of the type 3 strains The agglutinogens responsible for the tendency of strains of certain biochemical types to be related were not restricted to these types but were widely shared Thus the present investigation showed no better serologic correlation to the biochemical types of genus *Micrococcus* than the former investigation by *Oeding* (16) had shown to *Micrococcus* species Further no indication of serologic types irrespective of the biochemical types was revealed

True serologic types based upon agglutination do not seem to exist in micrococci If serologic characterization should be wanted this can probably be achieved by means of absorbed factor sera giving the pattern of agglutinogens demonstrable similar to the typing of *Staph aureus* (15) There may be a practical interest for this within the infectious type 3 strains

The indirect haemagglutination experiments confirmed the close antigenic relationship of the *Micrococcus* strains The strains evidently have several shared antigens sensitizing normal and tanned sheep erythrocytes to agglutination in *Micrococcus* immune sera These antigens are to a large extent shared also by *Staph aureus* It has earlier been demonstrated (1) that *Staph aureus* and *Staph epidermidis* share such antigens which seem to be rather broad in their distribution That *Staph aureus* also have sensitizing antigens with a high degree of specificity has been demonstrated by *Grov* (9) This may also be true of sensitizing antigens present in micrococci

The present results disagree with those of *Oeding* (16), which showed that *M luteus*, *M roseus*, and *M conglomeratus* strains had no or only a faint ability to sensitize sheep cells to agglutination in *Staph aureus* Cowan I serum whereas *M varians* strains had this ability In the present investigation only the red pigmented type 8 strain 1474 did not sensitize sheep cells to agglutination in the Cowan I serum The discrepancy must apparently be explained by the selection of strains

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ANTIGENIC STUDIES OF GENUS *MICROCOCCUS*

2 *Double Diffusion in Agar Gel with Particular Emphasis on Teichoic Acids*

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Fifty-six *Micrococcus* strains representing all the 8 biochemical types were tested on double diffusion in agar gel against *Micrococcus* immune sera and reference antigen/antibody systems Eighty per cent of the strains were found to have polysaccharide A$_\beta$C The polysaccharide A$_\beta$ precipitin line is produced by wall ribitol teichoic acid in which β linked N acetylglucosamine is the specific determinant The structure determining the polysaccharide C activity is unknown

In a preceding paper (8) *Micrococcus* strains representing the 8 biochemical types of *Baird Parker* (3) were found to share agglutinogens to a wide extent Agglutinogens characteristic of the biochemical types were not demonstrated and there was little, if any correlation between biochemical types and serologic results The present report gives the results of the precipitation studies

A polysaccharide substance containing ribitol teichoic acid was isolated from two strains of micrococci (originally classified as *Staphylococcus epidermidis*) by *Losnegard* & *Oeding* (14) On double diffusion in agar the polysaccharide gave two precipitin lines One line was confluent with the line produced by polysaccharide A (poly A$_\beta$) of *Staph aureus* (β N acetylglucosaminyl ribitol teichoic acid) the other precipitin line was designated C (15) The same precipitin pattern (poly A$_\beta$C) was demonstrated in several strai[n] isolated from the urine *Baddiley et al* (2 isolated wall teichoic acids from sever[al] strains of micrococci and characterized the[m] chemically Micrococcal strains were devide[d] into three groups, one of which had a ribit[ol] teichoic acid with N-acetylglucosaminyl re[si]dues

MATERIAL AND METHODS

Strains Fifty six strains of micrococci representin[g] all the 8 biochemical types of *Baird Parker* (3 were included in the study Thirty-one strains be longed to type 3 which was studied with particula[r] interest due to its reported ability to cause urina[ry] infections None of the strains attacked gluco[se] anaerobically (20) The origin and the chara[c]teristics of the strains have been given previousl[y] (8)

Sera Immune sera against three type 3 strai[ns] and against one strain of each of the other bio chemical types were produced by intravenous i[n]jections of formalin killed bacteria into rabbits (8 The strains used for immunization are referred t[o] below as the 'type strains'

Double diffusion in agar The technique was th

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same as that described in (11) Agar Noble (Difco) 1 5 per cent, was used Both extracts of the strains and thick suspensions of 18 hr cultures were tested against undiluted immune sera

Reference Systems

Poly A_β/serum *Staph aureus* Wood 46 (9)
Poly A_α (poly 263)/serum *Staph aureus* 263 (12)
Protein A/serum *Staph aureus* Cowan I (7)
Poly B_α (poly B)/serum *Staph epidermidis* 1254 (15)
Poly $A_\beta C$ (or *Microc* 3519 bacteria)/serum *Microc* 3519 (15)
Antigen D (*Staph aureus* 263 bacteria)/serum *Staph aureus* 263 (13)

RESULTS

When the 'type strains" were tested against the *Micrococcus* immune sera in agar diffusion the type 2 system gave 3 precipitin lines, the type [illegible] system 1 line, and the other systems 2 lines Table 1 shows which precipitin lines could be identified in the 10 "type strains" when they were tested against the reference systems Some lines were not identified The poly A line produced by 7 of the "type strains" fused completely with the reference poly A_β line Further, absorptions of the poly A_β reference serum (anti *Staph aureus* Wood 46) with 4 of these strains resulted in disappearance of the poly A_β line

The results showed that "type strains' 2, 3, 4, 5 and 7, all have poly $A_\beta C$, "type strains' 1 and 6 have poly B_α, whereas "type strain" [illegible] has none of these antigens

All the 56 *Micrococcus* strains were tested for the presence of the reference precipitinogens Table 2 shows that the vast majority of the strains has both the A_β and the C precipitinogen, a few strains seem to have either A_β C or B_α, and 4 strains have none of these precipitinogens The precipitinogens poly A_α, antigen D and protein A were not demonstrated in any strain Table 3 gives the correlation between the demonstrated precipitinogens and the biochemical types to which the strains belong It is seen that in 2 of 3 type 8 strains none of the precipitinogens was demonstrated

TABLE 1 *Identification of Precipitin Lines on Double Diffusion in Agar of the 10* Micrococcus *'Type Strains' Against the Reference Systems*

'Type strains	Poly A_β	Poly C	Poly B_α	Poly A_α, antigen D protein A
4036 (1)	—	—	+	—
4275 (2)	+	+	—	—
s/m 26 (3)	+	+	—	—
Bn U99 (3)	+	+	—	—
9145 (3)	+	+	—	—
1839 (4)	+	+	—	—
10653 (5)	+	+	—	—
1463 (6)	—	—	+	—
5350 (7)	+	+	—	—
1474 (8)	—	—	—	—

Numbers in brackets biochemical type
— negative

TABLE 2 *Presence of Reference Precipitinogens in 56* Micrococcus Strains

	No of strains	In per cent
Poly $A_\beta C$	45	[illegible]
Poly A_β	3	5
Poly C	1	2
Poly B_α	2	4
Poly $B_\alpha C$	1	2
No poly A_β, B_α or C	4	7
All	56	100

TABLE 3 *Distribution of Reference Precipitinogens within Biochemical Types*

	Biochemical type							
	1	2	3	4	5	6	7	8
Poly $A_\beta C$	6	5	29	1		1	3	
Poly A_β			1	1	1			1
Poly C								
Poly B_α	1					1		
Poly B_α C		1						
No poly A_β, B_α or C			1			1		2
No of strains	7	6	31	2	1	3	3	3

DISCUSSION

The present investigation confirms the report of *Losnegard & Oeding* (15) that antigens

giving rise to the precipitin lines $A_{\beta}C$ are present in *Micrococcus* cell walls The reference strain (3519) used for the identification of the $A_{\beta}C$ lines was first classified as *Staph epidermidis* but later shown to be a true *Micrococcus* The isolated wall polysaccharide contained ribitol teichoic acid with glucosaminyl residues (14), which is in accordance with the findings of *Baddiley et al* (2) for one group of micrococcal strains Furthermore wall ribitol teichoic acid with glucosaminyl residues isolated from *Staph saprophyticus* NCTC 7292 (4) and *Micrococcus* 234 (19), gave the same $A_{\beta}C$ precipitin lines Strain 7292 has later been shown in this laboratory, and by other authors, not to produce acid from glucose anaerobically

Of the 56 *Micrococcus* strains tested in the present investigation 45 (80 per cent) were shown to have poly $A_{\beta}C$ Four additional strains either produced the poly A_{β} line or the poly C line These strains may also have poly $A_{\beta}C$, the non reacting antigen being present in too small amounts to be detected Poly $A_{\beta}C$ thus appears to be characteristic wall constituents of the majority of *Micrococcus* strains although, in a previous investigation (18), only 3 out of 18 *Micrococcus* strains produced the poly A_{β} line *Baddiley et al* (2) reported that a second group of micrococci contains complex glycerol teichoic acids and a third group no wall teichoic acid According to the serologic examination seven strains of the present material belonged to *Baddiley et al*'s group 2 or 3 *Micrococcus* strains of these groups thus seem to be far less frequent than strains containing glucosaminyl ribitol teichoic acid No correlation was found between the content of precipitinogens and the biochemical types, although it was interesting that in two of the three type 8 strains none of the reference precipitinogens was detected serologically

The letter A of poly AC was chosen by *Losnegard & Oeding* (15) because one of the two precipitin lines fused completely with the poly A_{β} line of *Staph aureus* This species has a wall ribitol teichoic acid, one antigenic determinant of which was shown in agar diffusion to be its β-N acetylglucosaminyl residues (10) The present investigation confirms the apparent identity of the poly A_{β} line produced by *Staph aureus* and that produced by *Micrococcus* strains (Fig 1) Both groups of organisms thus appear to have one identical antigenic determinant, *i e* β-N-acetylglucosamine, in their teichoic acids Whether *Micrococcus* teichoic acid, like the *Staph aureus* teichoic acid, in addition has molecules with serologically active α-linkages of its aminosugar, remains to be shown Although this precipitinogen (poly A_{α}) was not demonstrated in the present investigation this does not exclude the presence of small amounts of α linked N acetylglucosamine

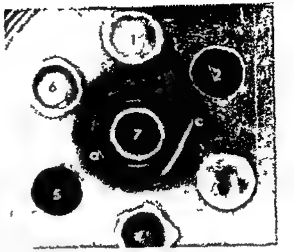

Fig 1 Double diffusion in agar showing the identity of the *Staph aureus* poly A_{β} line and the *Micrococcus* poly A line (a) Poly C line = c Poly AC reference serum in 7, reference AC bacteria in 1 and 4 Purified poly A_{β} from *Staph aureus* Wood 46 in 2 and 5

The antigen responsible for the C line (Fig 1) is unknown It is found in *Micrococcus* not in *Staph aureus*, thus distinguishing the two groups of organisms on agar gel precipitation It occurs together with poly A_{β} when live bacteria polysaccharide extracts and teichoic acid preparations are tested serologically Poly C may be an antigenic determinant of another teichoic acid, or more likely, of a polysaccharide regularly present in micrococci beside the ribitol teichoic acid If a sugar or an amino sugar determines the spe-

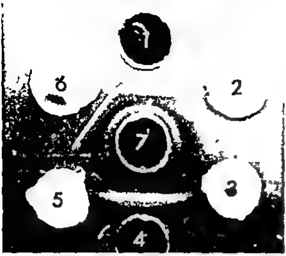

Fig 2 Double diffusion in agar of 4 *Micrococcus* strains against the poly B_{α} reference system (serum in 7, poly B_{α} in 1 and 4) Strain 1463 (6) is seen to produce the poly B_{α} line

cificity of poly C, then the usually strong C line would indicate that the sugar should be readily demonstrable in purified preparations Glucose has been detected in some *Micrococcus* polysaccharide or teichoic acid preparations (14, 19, 2, 6), but not regularly, and in only small amounts The possibility cannot be excluded that glucosamine is connected not only with the poly A_{β} specificity in AC, but also with the poly C specificity (6)

Six out of 48 *Staph epidermidis* strains were found by *Aasen & Oeding* (1) to produce the poly A_{β} line one of them also the poly C line Among the present strains of micrococci 3 produced the poly B_{α} line (Fig 2) This precipitin line is determined by the α linked sugar residues of the *Staph epidermidis* glucosyl glycerol teichoic acid Exceptions to the presence of the characteristic teichoic acid, like those referred to above may be the results of a deficient primary classification The two materials had been classified according to their ability or inability to attack glucose anaerobically The standard method (20) has been criticized by various authors because it does not satisfactorily distinguish staphylococci from micrococci (17, 5 16) It is very likely that the type of teichoic acid is a more fundamental character than glucose fermentation and should be given more weight in the classification

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AN ALTERNATIVE PROCEDURE FOR PRODUCTION OF ANTI-Lp(a) SERUM

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An immunization schedule different from the one previously used for the production of anti Lp(a) serum is described Rabbits were given partially purified Lp(a) lipoprotein prepared from relatively large amounts of Lp(a+) serum in the foot pads and intramuscularly The antisera obtained after absorption were specific and of high titres By repeated immunizations considerable quantities of antisera were obtained

The genetic Lp polymorphism of human serum β-lipoprotein (*Berg* 1963) comprises two phenotypes, Lp(a+) and Lp (a—) They can be identified by the use of absorbed hetero immune sera High quality antisera are a prerequisite for work with this genetic system The material of choice for immunization of animals has been lipoproteins prepared from serum of Lp(a+) individuals by chromatography on hydroxylapatite columns Of the immunization techniques tried by *Berg* (1965) only the intravenous route of injection led to useful antisera

Although the Lp(a) antigen is closely related to β lipoprotein it is present in a higher density class *Wiegandt et al* (1968) found the antigen in the density class 1 050–1 125 g/ml

We now report an alternative method for production of Lp(a) antisera of high quality

Received 5 x 71

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MATERIALS AND METHODS

Human sera Healthy Lp(a+) and Lp(a—) blood donors belonging to our reference panel were bled after fasting overnight In addition *210 blood* samples from healthy donors were kindly placed at our disposal by Dr *H Heisto* Blood Bank and Department of Immunohaematology Oslo City Hospital

Reference antisera Serum from rabbit K 57 from the original study on the Lp system (*Berg* 1965), and serum R 39, another standard Lp(a) antiserum produced at our Institute were used

Preparation of antigen for immunization Lp(a+) serum was fractionated by centrifugation at 105,000 × *g* in a Beckman *Spinco* Model L2-65B ultracentrifuge The densities were adjusted with solid KBr The centrifugations were performed for 22 hours for the density 1 063 g/ml and for 45 hours for the density 1 125 g/ml The final supernatant fluid containing the lipoproteins of density 1 063 1 125 g/ml was dialyzed against 0 85 per cent (w/v) saline containing 5 mg/l of Na EDTA, and used for immunization Antigen solutions to be used for booster doses were kept at —27° C

Analyses of rabbit immune sera Testing of the antisera and absorption experiments were performed as described by *Berg* (1965) Agar purum (Behringwerke AG Marburg) was used for the double diffusion tests

EXPERIMENTS AND RESULTS

Three albino rabbits (R 52, R 53, and R 54) weighing approximately 3 kg were each given 1 ml of antigen prepared from 12 ml serum, in the foot pads and in multiple intramuscular sites The antigen was mixed with 1 ml of Freund s complete adjuvant (Difco laboratories, Detroit) After 3 weeks the same amount of antigen without adjuvant was given intramuscularly and subcutaneously Seven days later blood was collected from the marginal vein of the rabbit's ear Additional injections (same amount of antigen) were given at intervals of 3–10 weeks, and the animals were bled 7 days after each booster dose

The antisera obtained from the immunized rabbits exhibited high titres against Lp(a+) sera after absorption with Lp(a—) serum, whereas absorption even with small amounts of Lp(a+) serum removed all precipitating capacity (Table 1) The majority of the precipitin bands appeared during the first 24 hours of incubation, and all bands were visible within 48 hours Once a precipitin band had developed, it was never observed to disappear again

To confirm that these antisera possessed the specificity anti Lp(a), they were compared with the reference antisera in double diffusion tests Reactions of identity between the precipitin bands were demonstrated Moreover, the antisera were found to be in complete agreement in the capacity to discriminate between Lp(a+) and Lp(a—) sera, when 210 sera from blood donors were tested

The effect on the quality of the antisera of giving the rabbits repeated booster doses is illustrated in Fig 1

DISCUSSION

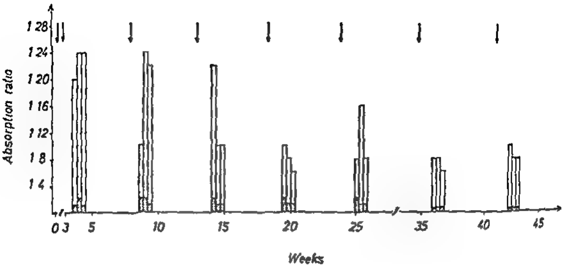
The method described resulted in high quality reagents, and we conclude that the combination of injections in the foot pads and in intramuscular sites of relatively large amounts of antigen can be recommended for the production of anti Lp(a) sera in rabbits In fact the antisera we have now produced are stronger than any we have made previously, and to our knowledge they are among the best ever produced They satisfy all reasonable requirements to the quality of Lp(a) typing reagents (*Berg* 1965)

By the technique we used for preparation of the antigen, almost all β lipoprotein is eliminated This may be an explanation why high titres of antibodies to common β lipoprotein antigens did not become a major problem with antisera obtained after repeated booster doses The α_1-lipoprotein present in our immunization materiel did not initiate the production of large amounts of antibodies to this protein Other 'unspecific" antibodies

TABLE 1 *Reaction Pattern Obtained with Absorbed Anti Lp(a) Serum and Serum from Lp(a+) and Lp(a—) Persons by Agar Gel Double Diffusion Experiments*

Immune serum from rabbit	Normal human serum of type	Ratio (volumes) Rabbit immune serum serum from Lp(a—)human donor									Ratio (volumes) Rabbit immune serum serum from Lp(a+) human do				
		2 1	1 1	1 2	1 4	1 8	1 16	1 24	1 32	1 40	2 1	1 1	1 2	1 4	1
R 52	Lp(a+)	+	+	+	+	+	+	(+)	—	—	+	+	(+)	—	—
	Lp(a—)	+	+	—	—	—	—	—	—	—	+	(+)	—	—	
R 53	Lp(a+)	+	+	+	+	+	+	+	(+)	—	+	+	(+)	—	—
	Lp(a—)	+	+	+	—	—	—	—	—	—	+	+	—	—	—
R 54	Lp(a+)	+	+	+	+	+	+	+	—	—	+	+	+	—	—
	Lp(a—)	+	+	—	—	—	—	—	—	—	+	+	—	—	—

\+ – visible precipitate   — = no visible precipitate



antigen injection

absorption ratios where immune serum reacts only with Lp(a+) human sera

absorption ratios where immune serum reacts with all human sera

Fig 1 Effect of prolonged immunization with human Lp(a) antigen The three columns represent immune serum obtained from rabbits R 52, R 53, and R 54, respectively, one week after each booster dose

were of low titres which showed no tendency to increase during the immunization course Therefore, even though the titres of specific antibodies to the Lp(a) antigen fell after repeated booster doses to approximately one third of the initial concentrations, the immune sera could easily be made specific by absorption in tubes with a good margin of safety Thus large quantities of specific anti-Lp(a) serum were obtained during the experimental period

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HUMAN INFECTIONS WITH *FRANCISELLA TULARENSIS* IN NORWAY

Development of a Serological Screening Test

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A sero epidemiological study of F tularensis infection has been performed in school children and hunters in some districts of Norway The results have revealed a considerable incidence of such infection in certain districts as judged by the chosen criteria The study has offered an opportunity to develop a modification of a slide agglutination technique suitable for the screening of large numbers of serum samples

In Scandinavia there has traditionally been much interest in tularemia One of the classical contributions on its occurrence in animals was thus made by *Horne* (7) Early observations on tularemia in humans were published by *Thjotta* (10, 11) During recent years there has however been only sparse reports of human cases in Norway (6) This is in definite contrast to the situation in Sweden, where extensive epidemics have occurred (1 9)

It has been generally accepted that the infection has its reservoir among warm-blooded animals notably rodents (8) Man can be infected in various ways Thus blood sucking arthropods such as fleas and ticks can carry the organism (2 3, 4, 5 12) Many unsolved questions remain however concerning the epidemiology of F tularensis infection and clearer understanding can obviously only be acquired through extensive studies involving also epizootological aspects

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It has been considered useful on this background to report the results of a sero-epidemiological investigation of selected population groups in some districts of Norway

The main purpose of the study has been to obtain information on the importance of F tularensis infection in the various districts from a public health point of view

A problem of particular interest in this connection is the relation between the incidence of F tularensis infection as judged by serological data and the occurrence of overt clinical tularemia

The investigation has offered an opportunity to develop a modification of a slide agglutination technique which facilitates the screening of large numbers of serum samples for epidemiological purposes

METHODS

Antigens Four different strains of F tularensis have been employed for the preparation of agglutination antigens

Strain designation	Abbreviation	Provided by
Jap Down	JAP	Veterinary GUNNAR HOLT, Veterinærinstituttet, Oslo
HN 63 (hare)	HARF	Professor SVERRE DICK HENRIKSEN, Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, Oslo
MRE 127 Str 503	503	Dr H M DARLOW, Microbiological Research Establishment, Porton UK
609 Str Schu S4	SCHU	Dr H M DARLOW, Microbiological Research Establishment Porton, UK

The antigens were prepared by harvesting glucose cystine rabbit blood agar cultures (18 hours at 37° C) into physiological saline The organisms were killed by adding 40 per cent formaldehyde to a final concentration of 0 5 per cent The suspensions were checked for sterility and washed three times in physiological saline From the stock suspension prepared in this way the final working suspensions were standardized colorimetrically (cfr below)

Tube agglutination The basic method used in this study was the Widal type tube agglutination The technique had to be modified for the present study by practical testing and adaptation of the single features the concentration of antigen the incubation time and temperature and the reading of results The tube agglutination method was used in the material of hunters suspected human cases and vaccinated persons It was furthermore used in part of the school children group

The antigen concentration found optimal under the conditions corresponded to a transmittance of 60 (Beckman colorimeter model C, wave length 524 $m\mu$) After extensive trials an incubation procedure of 37° C for 18–20 hours was chosen

The final serum dilutions after the addition of antigen were 20 40, 80, 160 etc (reciprocal values)

The results were read according to the following criteria

+ + + +	abundant agglutination, coarse flakes
+ + +	moderate agglutination, coarse flakes
+ +	abundant agglutination fine grains
+	moderate agglutination fine grains
(+)	trace of agglutination
—	no agglutination

The results were read by naked eye against a dark background, in questionable cases by aid of a hand lens The highest serum dilution yielding a + (one plus) reaction was regarded as titre value

Slide agglutination A slide agglutination procedure was used in parallel with the tube agglutination for the purpose of comparison The slide agglutination was employed in the whole material In accordance with the other workers a titre of 80 or above has been regarded as positive (1)

Prior to the application of the former technique basic trials were performed on all essential features varying them one by one to find the combination most suited Trials have thus been made concerning volume of reactants and technique of quantitation concentration of antigen, dilution of antiserum time of incubation, and reading of results An antigen concentration corresponding to a transmittance of 25 (cfr tube agglutination) was found most suitable

The agglutinations were carried out on special glass plates with black burnt in rings (Hyland Laboratories, USA) Equal volumes of antigen and serum dilution (one drop of each) were mixed one sample in each ring The plates were placed in a moist chamber on a slow motion shaking machine (A Buhler no 150100) in a 37° C incubator room and shaken for 25 minutes After about 5 minutes on the desk reading was performed against an illuminated background by aid of a lens otherwise according to the same criteria as the tube agglutination

To facilitate the reading the antigen was stained blue by adding one or two drops of Loefflers methylene blue per 10 milli litres of antigen suspension

Because the slide technique was intended as a screening procedure all agglutination reactions including the weakest, (+) were recorded In all instances the sera were used without previous inactivation

When used in the slide agglutination all sera were diluted 1 10 During the early phase of the study undiluted sera were employed in parallel this practice was abandoned due to the occurrence of a high proportion of reactions which could not be verified by tube agglutination (cfr Results and Discussion)

MATERIAL

A) Epidemiological Survey

1) School children Districts within four regions of Southern Norway were selected Telemark T■

Fig 1

a) Hallingdal and Trondelag Study areas were chosen where the livelihood of the indigenous population was largely agriculture and forestry and all areas had had previous reports of tularemia in animals and/or man (6 10) although no recent cases have been recorded in Hallingdal

Because of school centralization it has been practicable to reach a majority of the age groups attending the last two classes (in some cases three) of the 9 year primary school which is obligatory The percentage attending the blood sampling was in all schools high on the average 74 per cent and in no school less than 62 per cent

The majority 85 per cent of those tested belonged to the age group 13 14 years The rest 15 per cent belonged to the age group 15–17 years The total number of children examined is 813 (cf the tables)

The geographical distribution is seen from Fig 1

2 *Hunters* The districts of Telemark and Trysil were chosen, and the groups included members of local game boards and/or hunters' organizations The general public showed considerable interest in this investigation and a total of 55 persons participated in this part of the survey

B) Evaluation and Comparison of Techniques

1) Recently diagnosed or suspected human cases of tularemia This group includes *11 persons* Some of the sera were placed at our disposal by The National Institute of Public Health, Bacteriology Department, the rest are sera submitted directly by local physicians and hospitals in various districts of Norway

2) Persons vaccinated against tularemia This group consists of *18 persons* belonging to the laboratory staff or otherwise related to the project They had all been vaccinated with live F tularensis vaccine manufactured by The National Drug Company, Philadelphia, USA With the exception of the sample 98GH, which had shown a low titre agglutination reaction before vaccination, all were negative prior to vaccination

RESULTS

The serological survey is presented in Tables 1, 2, and 3 Tables 2 and 3 include all samples showing detectable agglutination of one or more of the four antigens The results have been arranged as far as possible according to increasing titre by tube agglutination

1) School children Table 1 shows the frequencies of agglutination of a titre of 80 or above of one or more of the antigens The frequencies when 20 and 40 are chosen as limits, are also shown There is apparently a geographical variation in the incidence of positive agglutinations Because of the low incidence level, the material does not however, allow statistically significant conclusions The individual positive results are reviewed in Table 2 The number of significant positive results is not great enough to permit conclusions concerning the over-all quantitative titre level of such results from district to district It appears from the table, however that the positive results from Trondelag show somewhat higher titres than those from the other districts

2) Hunters Table 3 shows the agglutination results of sera from hunters

TABLE 1 *Frequency of Positive Tube Agglutination Results*) in Sera from School Children*

District	No of samples	Pos No	Titre ≧ 20 Per cent	Pos No	Titre ≧ 40 Per cent	Pos No	Titre ≧ 80 Per cent
Telemark	307	10	3.2	8	2.6	5	1.6
Trysil	202	0	0	0	0	0	0
Hallingdal	115	3	2.6	2	1.7	1	0.9
Trøndelag	191	9	4.7	8	4.2	5	2.6
Total	815	22	2.7	18	2.2	11	1.3

*) in one or more antigens

TABLE 2 *Positive Slide and Tube Agglutination Results of Sera from School Children with Antigens prepared from 4 Different Strains of* F tularensis

District	Serum no	JAP		HARE		503		SCHU	
		Tube	Slide	Tube	Slide	Tube	Slide	Tube	Slide
Telemark	126	—	—	—	—	—	—	20	+
	139	—	—	—	—	—	—	20	+
	150	—	—	—	—	—	—	40	+
	168	—	+	20	(+)	—	+	40	+ +
	38	40	+ +	40	+ +	40	+ +	40	+ +
	284	40	+ +	40	+ +	40	+ +	80	+ +
	302	40	+ +	80	+ +	40	+ +	80	+ +
	142	40	+ +	160	+ +	80	+ +	160	+ +
	222	80	+ +	80	+ +	80	+ +	***)	+ +
	258	80	+ +	160	+ +	160	+ +	160	+ +
Hallingdal	799	20	(+)		(+)		(+)		(+)
	800	40	(+)		(+)		(+		(+)
	794	80	(+)		(+)		(+)		(+)
Trøndelag	718	20	—		—		—		(+)
	709	40	—		—		—		(+)
	701	40	(+)		(+)		—		(+)
	597	40	+ +		+ +		+ +		+ +
	574	80	—		—		—		(+)
	587	160	+ +		+ +		+ +		+ +
	708	160	+ +		+ +		+ +		+ +
	694	320	+ + +		+ + +		+ + +		+ +
	662	1280	+ + +		+ + + +		+ + +		+ +

***) no serum left

TABLE 3 *Agglutination Results of Sera from Hunters*

District	Serum No	JAP		HARE		503		SCHU	
		Tube	Slide	Tube	Slide	Tube	Slide	Tube	Slide
Telemark**)	10	20	—	*)	—	*)	—	*)	—
	15	20	—	*)	+	*)	—	*)	+
	26	160	+	160	+	80	—	160	—
Trysil***)	844	160	+ + +	160	+ + +	160	+ + +	160	+ + +
	845	320	+ + +	320	+ + +	160	+ + +	320	+ + +

*) not tested
**) 26 persons
***) 29 persons

TABLE 4 *Agglutination Results of Sera from Recently Diagnosed or Suspected Human Cases of Tularemia (11 cases)*

Serum no	JAP		HARE		503		SCHU	
	Tube	Slide	Tube	Slide	Tube	Slide	Tube	Slide
53	—	—	—	—	—	—	20	—
59	20	—	20	—	20	—	20	—
23	20	—	*)	—	*)	—	20	—
41GR	20	—	*)	+	*)	—	*)	+
86C	40	+	40	+	40	(+)	80	+ +
99SP	40	+ + +	80	+ + +	40	+	80	+ +
49	80	+ +	40	+ +	80	+ +	80	+ +
89SJ	160	+ + +	80	+ + +	80	+ +	80	+ + + +
1/70 OJ	160	+ + +	160	+ + +	160	+ +	160	+ +
54EO	2560	+ + + +	640	+ + + +	1280	+ + + +	1280	+ + +
52KP**)	2560	+ + + +	1280	+ + + +	640	+ + + +	1280	+ + + +
33KP**)	2560	+ + + +	1280	+ + + +	1280	+ + + +	1280	+ + +
58KP**)	2560	+ + + +	2560	+ + + +	1280	+ + + +	2560	+ + + +
8KP**)	5120	+ + + +	1280	+ + + +	1280	+ + + +	1280	+ + + +
20KP**)	5120	+ + + +	1280	+ + + +	1280	+ + + +	1280	+ + + +

*) no serum left.
**) different samples from the same patient

TABLE 5 *Agglutination Results of Sera from Persons Vaccinated against Tularemia*

Serum no	JAP		HARE		503		SCHU	
	Tube	Slide	Tube	Slide	Tube	Slide	Tube	Slide
77IMB	**)	—	**)	—	**)	—	**)	—
72AG	—	—	—	—	—	—	—	—
101EM	—	(+)	20	(+)	—	(+)	20	(+)
98GH	40	+ + +	40	+ + +	40	+ +	40	+ + +
96EMB	40	+ +	40	+ +	40	(+)	80	+
6/70HS	40	+ + +	80	+ + +	40	+ +	80	+ + +
93EA	80	+	80	+	40	—	80	+ +
3/70EM	80	+ + +	80	+ + +	80	+	80	+ +
5/70KV	80	+ + + +	80	+ + + +	80	+ + +	80	+ + + +
7/70PS	80	+ + + +	80	+ + + +	80	+ + + +	80	+ + + +
76LH	80	+ + + +	80	+ + + +	80	+ + + +	80	+ + + +
78TO	80	+ + +	80	+ + +	160	+ +	160	+ + +
75RHH	160	+ + + +	80	+ + + +	80	+ + +	160	+ + + +
97EA	160	+ + + +	160	+ + + +	160	+ + + +	80	+ + + +
ADP***)	160	+ + + +	160	+ + + +	160	+ + + +	160	+ + + +
4/70AMLS	160	+ + + +	160	+ + + +	160	+ + +	320	+ + +
94OL	320	+ + + +	160	+ + + +	160	+ +	160	+ + + +

**) not tested
***) vaccinated earlier

The Evaluation and Comparison of Techniques

1) *Recently diagnosed or suspected cases of tularemia* Table 4 reviews the results in this group Out of a total of 11, 7 are positive when a titre of 80 is chosen as limit

2) *Persons vaccinated against tularemia* Table 5 reviews the results Out of a total of 17, 13 are positive using a titre limit of 80

Using a titre limit of 40, 14 are positive The average titre level of the positive samples is similar to the level in the groups of school

TABLE 6 *Correlation of Slide Agglutination and Tube Agglutination Results*

Slide agglutination	JAP Tube agglutination —	20	40	80	160	320	640	1280	2560	5120	Total
—	5	6	1	1							13
(+)	1	1	2	1							5
+	1		1	1	1						4
+ +			6	2	3						11
+ + +			3	2	3	2		1			11
+ + + +				3	4	1			4	2	14
Total	7	7	13	10	11	3		1	4	2	58

Slide agglutination	HARE Tube agglutination —	20	40	80	160	320	640	1280	2560	5120	Total
—	5	1									6
(+)		2									2
+			1	1	1						3
+ +			4	2	2						8
+ + +			1	5	2	1					9
+ + + +				4	4		1	4	1		14
Total	5	3	6	12	9	1	1	4	1		42

Slide agglutination	503 Tube agglutination —	20	40	80	160	320	640	1280	2560	5120	Total
—	5	1	1	1							8
(+)	1		2								3
+	1		1	1							3
+ +			5	4	4						13
+ + +				2	3						5
+ + + +				2	2		1	5			10
Total	7	1	9	10	9		1	5			42

Slide agglutination	SCHU Tube agglutination —	20	40	80	160	320	640	1280	2560	5120	Total
—	1	3			1						5
(+)		1									1
+		2	1	1							4
+ +			2	7	3						12
+ + +			1	1	2	2		2			8
+ + + +				5	3			3	1		12
Total	1	6	4	14	9	2		5	1		42

children and hunters and thus considerably lower than in the sera from recently diagnosed or suspected cases of tularemia

In both groups (1) and 2)) there is a good correlation between the results obtained by either of the two techniques With one exception all samples with a titre of 40 and above in tube agglutination show positive results also in the slide technique in all four antigens (93EA)

All sera which have been tested by both methods including all sera which showed detectable agglutination of any antigen, have been incorporated in Table ■

DISCUSSION AND CONCLUSION

The main result of the present study ■ that it has revealed the presence of a considerable incidence of F tularensis infection as judged by serological criteria The question of possible cross reactions may be raised Such reactions may be expected to occur especially in the lower titres General epidemiological evidence would, however, seem to suggest that reactions of this kind are not likely to change the picture radically, although little is known concerning the occurrence of human infections by microorganisms which might be antigenically related to *F tularensis* except for brucellosis which does not occur in Norway at present

As concerns the occurrence of overt clinical tularemia in relation to the serological results, the study seems to indicate ■ difference between the school group and the hunters' group In the latter group all three individuals showing positive agglutination are known to have had tularemia (cfr Table 3) In the school groups no information of clinically diagnosed tularemia has been obtained during the collective examination in the schools Further investigations are, however, under way based on direct interviews of each family in question

The purpose of developing a slide agglutination has been to obtain a method for future epidemiological screening of large numbers of sera Consequently the slide agglutination has to fulfill one basic requirement i e a sufficiently high sensitivity level If titres of 80 and above by tube agglutination are regarded as specific, it ■ seen from Table 6 that the incidence of slide technique failure is 1 in 58 using the Jap antigen, 1 in 42 in the 503 and Schu antigens, whereas the Hare antigen does not show slide technique failure at all in the 42 instances recorded

It may thus be concluded that the findings point to F tularensis infection as one of considerable importance in Norway The problem of the relation between such an infection as judged by serological criteria alone, and overt clinical tularemia, needs, however, further elucidation

We are indebted to Professor *S D Henriksen*, Amanuensis *G Holt* Dr *J Kvittingen*, Director *C Lerche*, Professor *A Semb Johansson* for advice and encouragement during the planning and implementation of the work We also want to express our gratitude to Medical Officers, School Authorities and parents, members of Hunters' Associations and Game Boards in the districts under study

We are finally indebted to Mrs *Ellen Marie Gruner* for excellent technical assistance

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ELECTRON MICROSCOPY OF STAPHYLOCOCCAL PROTEIN A REACTIVITY AND SPECIFIC ANTIGEN-ANTIBODY REACTIONS

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The quantitative and qualitative difference between protein A reactivity of *Staphylococcus aureus* and the specific adsorption of homologous antibodies was demonstrated by means of ferritin labelled rabbit serum globulins and electron microscopy Protein A antigen seems to reside in the outermost layer of the cell wall and is evenly distributed over the whole cell

In 1964 (7) it was suggested that the reactivity between certain strains of *Staphylococcus aureus (S aureus)* and fluorescein isothiocyanate (FITC) labelled globulins was not a specific reaction in the usual sense of an antigen antibody reaction, but rather a non-immunological affinity between the bacterial cells and globulins This hypothesis arose from results obtained in the fluorescent antibody test (FAT) using FITC labelled globulins from normal and immune animals as described by *Lind* (9), and it was later confirmed by immunoelectrophoretic analyses in combination with absorption experiments (11) Supported by the analogous findings of *Forsgren & Sjöquist* (1 2), it was concluded that this reactivity was due to a strong affinity between the staphylococcal surface antigen protein A and γG globulins

In the present work it has been attempted to demonstrate by electron microscopy the protein A reactivity of staphylococci, as well as some specific antigen-antibody reactions Furthermore, investigation has been made of possible differences between the cell wall structure of strains which produce large amounts of protein A (pA pos strains) and those which produce very little or no protein A (pA neg strains) In previous reports such strains were designated "*reactive*" and "*non reactive*", respectively (9, 10 11)

MATERIAL AND METHODS

Bacterial strains Two pA pos (4972/62 63 8341/61–62) and two pA neg (20036/61 62 E 1969) strains of *S aureus* were used These had been examined previously for their ability to adsorb serum protein components (11) Three strains of *Neisseria gonorrhoeae* (*N gonorrhoeae*) (19827/40, 11415/40 17924/63–64) were also examined

Sera Preparation of rabbit antigonococcal and rabbit antistaphylococcal sera has been described previously in detail (8 9) The following sera were labelled with ferritin 1) a pool of eight rabbit antigonococcal sera prepared against the same pool of formalin treated gonococci (seven strains including three strains used in the experiments to be

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Received 13 x 71

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described), 2) a pool of eight normal sera originating from the rabbits later immunized with staphylococci, 3) a pool of four rabbit antisera against five pA pos (*reactive*) staphylococcal strains described previously (9), and 4) a pool of four rabbit antisera against five pA neg (*non reactive*) strains of *S aureus*

In the indirect staining procedure, samples of the serum pools described were used in the first step An antiserum homologous to *N gonorrhoeae* 17924 was also employed Two sheep antisera against rabbit serum globulin were obtained from Sylvana Chemical Company, Orange, N J, U S A, and through the courtesy of [illegible] *Mansa*, Department of Biophysics, Statens Seruminstitut, these were labelled with ferritin

In supplementary experiments, a rabbit antiferritin serum and a pool of normal rabbit sera were used, both kindly supplied by Joan M Rhodes, International Escherichia Centre, Statens Seruminstitut

Labelling with ferritin Ferritin (cadmium free) was obtained from *Pentex*, Illinois, U S A A crude γG globulin was prepared by precipitation of the rabbit serum three times with cold ammonium sulphate (one third saturation) at pH 7 8 (3) The labelling was performed essentially according to the method described by *Singer & Schick* (20) using toluene, 2,4 di isocyanate as coupling reagent

Gonococcal complement fixation test The test was performed according to *M Kristensen* (see 8) The results are given as $\log_{10}$ to the reciprocal value of the lowest serum concentration giving 60 per cent haemolysis

Indirect haemagglutination test for demonstration of staphylococcal antibodies For reasons given in a previous paper (9), this test was chosen for measuring the content of staphylococcal antibodies in rabbit sera before and after immunization The technique was described in detail in that paper The results are recorded as $\log_{10}$ to the reciprocal value of the lowest serum concentration giving a one plus reaction

Immunoelectrophoresis The micromethod of *Scheidegger* (18) was used, with the modifications described previously (11)

Fluorescent antibody test (FAT) The method for labelling globulin with fluorescein isothiocyanate (FITC) and the performance of the test were described previously (8)

Preparation of cell suspensions for electron microscopy Strains of *S aureus* were grown at 36° C on broth peptone agar containing 10 per cent horse blood Strains of *N gonorrhoeae* were grown on broth peptone agar containing 25 per cent ascitic fluid and were incubated at 36° C in a moist atmosphere containing 10 per cent carbon dioxide After 14–18 hours growth the cells were harvested in a 3 per cent formalin solution In the first experiments a yeast extract, sodium acetate, peptone medium (0 3 per cent, 0 05 per cent and 0 3 per cent, respectively, of the Difco products) (YAP medium) was used for dilution and in the later experiments phosphate buffered saline (PBS pH 7 2 (8)) From these stock suspensions 10 ml portions of known density (10^{10} cells per ml or 3×10^{9} cells per ml) were prepared in the formalin solution. The suspensions were left for half an hour at room temperature and centrifuged for 5 minutes at 7,000 *g*

Treatment of cells with ferritin labelled globulin a) *Direct technique* The pellets of formalin treated cells were resuspended in ferritin labelled globulin (diluted $^1/_{10}$ in YAP medium) by gentle treatment in a *Vortex* mixer The reaction mixtures were incubated in 37° C water bath for 45 minutes after which 1 ml of 1 per cent osmic acid in barbiturate acetate buffer pH [illegible] 1 (15) was added to each tube for prefixation Immediately afterwards 5 ml YAP medium was added The cells were resuspended by treatment in the *Vortex* mixer *b) Indirect technique* The pellet of formalin treated cells was resuspended in unlabelled serum (diluted $^1/_{10}$ in PBS pH 7 2) and incubated for 45 minutes in 37° C water bath The cells were spun down and washed three times in PBS pH 7 2 and resuspended in ferritin labelled sheep antirabbit globulin (undiluted diluted $^1/_{10}$ and $^1/_{100}$), then incubated for 45 minutes in 37° C water bath After centrifugation the cells were washed twice with PBS pH 7 2 and finally resuspended in 10 ml YAP medium 1 ml of 1 per cent osmic acid in barbiturate acetate buffer pH 6 1 was added to each tube for prefixation

Electron microscopy After prefixation with osmic acid for 10 20 minutes at room temperature, the cell suspensions were centrifuged at 2500 *g* for 10 minutes The pellets were enrobed in agar at 45° C (1 5 per cent agar in barbiturate acetate buffer at pH 6 1) After hardening of the agar blocks of about 1 mm³ were cut and fixed overnight at room temperature in 1 per cent osmic acid to which 0 1 per cent YAP medium was added The blocks were washed briefly in barbiturate buffer and

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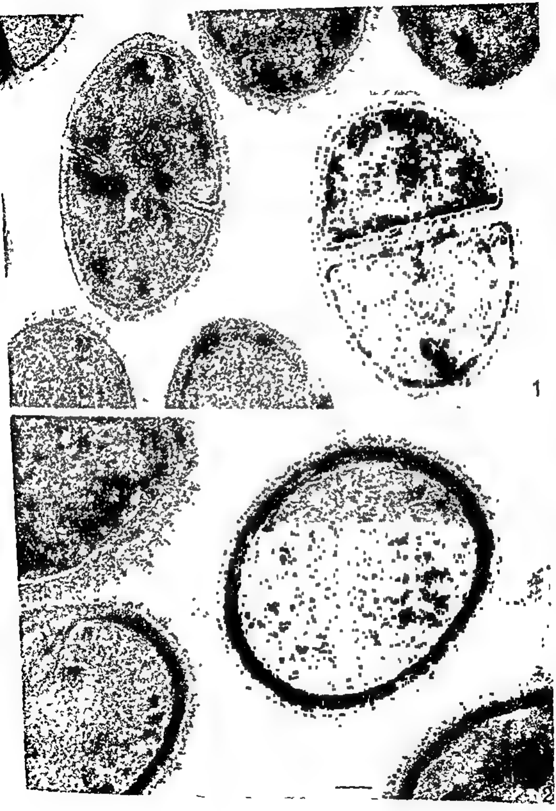
Text to figures

Unless otherwise stated the direct technique was used and the cells were treated with ferritin labelled globulin in dilution $^1/_{10}$ All sections illustrated were post stained with lead hydroxide The bar on each micrograph represents 0 1 *µm*

Fig 1 Untreated cells from a pA neg strain of *S aureus* (20036) Magnification 90 000 ×

Fig 2 Cells from a pA pos strain of *S aureus* (8341) treated with ferritin labelled normal rabbit globulin Note the uniform dense layer of ferritin labelled protein covering each cell Magnification 90 000 ×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treated for 1 hour with 2 per cent barbiturate buffered uranyl acetate. Finally they were dehydrated by acetone and embedded in Vestopal W (16). Sections were obtained on the LKB ultrotome I microtome and were examined with a Philips EM 200 electron microscope. Exposures were made on Kodak fine grain positive film at a primary magnification of 9 000 x. Some sections of all preparations were post-stained with solutions of lead hydroxide prepared according to method A of Karnovsky (4).

RESULTS

Effect of preparation procedure on protein A reactivity. The procedure described for preparation of cell suspensions for electron microscopy was followed except that 1) the cells were incubated with FITC labelled rabbit or sheep globulins (originating from the same serum pools as those used for ferritin labelling) and 2) that the cells were finally resuspended in a minute volume of PBS pH 7.2. From these suspensions smears were made on microscope slides and examined by fluorescence microscopy. The pA pos staphylococci fluoresced just as brilliantly as those in control smears prepared from plate cultures and stained according to the usual FAT procedure. The gonococci were stained less uniformly than the controls but undoubtedly a binding of labelled globulins occurred. The results obtained by direct and indirect staining techniques were identical. Thus, it was ensured that formalin treatment, washings, incubation and autoagglutinability (9) did not impair the protein A reactivity or antigen antibody reactions essentially.

Effect of ferritin labelling on specific antibody reactions. The specific antibody activity of the ferritin labelled globulins was evaluated by means of complement fixation (gonococci), indirect haemagglutination test (staphylococci) and immunoelectrophoretic analysis (rabbit serum globulins). In contrast to labelling with FITC, labelling with ferritin resulted in a decrease in antibody titre. The antigonococcal titre fell from 3.46 $\log_{10}$ to about 2 $\log_{10}$ (the conjugates were weakly anticomplementary). The antistaphylococcal titre fell from 4.00 to 3.70 $\log_{10}$. Antibodies against rabbit serum were not measured quantitatively, but specific precipitates were demonstrated by immunoelectrophoretic analysis before and after labelling. In addition, a positive reaction in the indirect staphylococcal haemagglutination test (2.81 $\log_{10}$) was obtained.

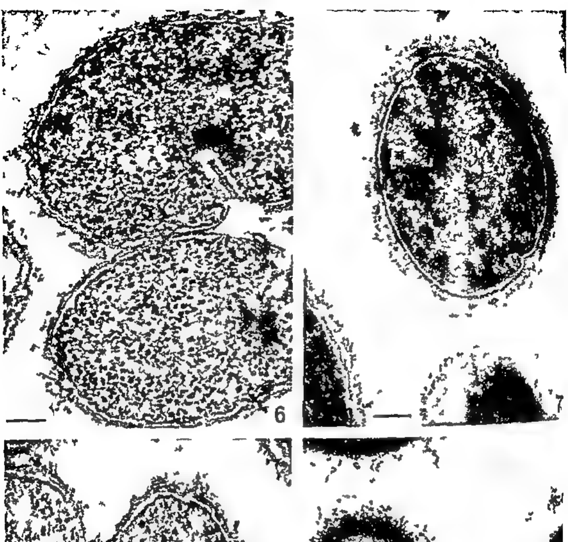
Fine structure of S. aureus. The cells showed all the characteristics of Gram positive bacterial strains (Fig. 1) (17). With the technique employed electron microscopy did not reveal any convincing morphological differences between untreated cells of pA pos and pA neg strains, particularly not as regards the structure of the cell wall.

Binding of ferritin labelled globulins (direct technique). a) pA pos strains of S. aureus. Fig. 2 shows *S. aureus* 8341 treated with ferritin labelled normal rabbit globulin. The corresponding serum did not contain antibodies against staphylococci demonstrable by the indirect haemagglutination test. A uniform dense layer of ferritin labelled protein covered the bacterial cells and the ferritin particles were seen in the outermost part of the protein coating. The demarcation line between the cell wall and the layer of protein was clearly visible. A completely identical picture was seen when a pA pos strain was treated with ferritin labelled heterologous immunoglobulin (rabbit antigonococcal globulin). Fig. 3 illustrates the treatment of strain 4972 with ferritin labelled homologous immunoglobulin. Also in this case a dense

Fig. 3. Cells from a pA pos strain of *S. aureus* (4972) treated with ferritin labelled rabbit antistaphylococcal globulin. Note that the ferritin particles show a tendency to accumulate in tufts. Magnification 90 000 ×.

Fig. 4. Cells from a pA neg strain of *S. aureus* (20036) treated with the same ferritin labelled normal rabbit globulin used in the experiment illustrated in Fig. 2. Only a few ferritin particles are seen between cells. Magnification 90 000 ×.

Fig. 5. Cells from the pA neg strain shown in Fig. 4 treated with ferritin labelled rabbit antistaphylococcal globulin. Little protein is attached to the cells and the localization seems fortuitous. Magnification 90 000 ×.



6 7 8 9

row of ferritin labelled globulin was seen on the surface of the cell but, in addition the ferritin particles showed a tendency to accumulate in tufts

b) pA neg strains of S aureus Fig 4 illustrates the reaction between strain 20036 and the same ferritin labelled normal rabbit serum as that used in the experiment illustrated in Fig 2 The surface of the cells is completely bare and only a few ferritin particles are seen between cells (In preparations from another strain (E 1369) a few cells covered with ferritin labelled globulin molecules were seen) Fig 5 corresponds to Fig 3 and shows a pA neg strain (20036) after treatment with ferritin labelled homologous immunoglobulin Surprisingly little protein was attached to the cells and the localization seemed fortuitous

c) N gonorrhoeae The appearance of antigen antibody reactions was investigated further by means of another antigen-antibody system *ci N gonorrhoeae* and ferritin labelled rabbit antigonococcal globulin Fig 6 shows that the adsorption of proteins is rather sparse with a tendency to become localized in tufts Fig 7 illustrates the reaction between the same ferritin labelled antigonococcal globulin and a pA pos strain of *S aureus*

In the experiments described, the binding of globulin, and especially that in the specific antigen antibody reaction, might have been impaired for the following reasons 1) the ferritin labelled globulins were used in a dilution of $^1/_{10}$ 2) the labelling procedure resulted in a fall in titre of the specific antibodies, and 3) the labelling with ferritin was not complete, perhaps less than 50 per cent In order to compensate for these disadvantages, two additional series of experiments were performed The first series included unlabelled antisera and undiluted ferritin labelled globulins and in the second the indirect technique was employed

Fig 8 shows the same gonococcal strain as that seen in Fig 6 treated with unlabelled homologous antiserum in dilution $^1/_{10}$ The layer of protein is more abundant and more evenly distributed than that observed in the corresponding preparation with ferritin labelled globulin diluted $^1/_{10}$ A tendency to become localized in tufts is still seen

Fig 9 shows a pA neg staphylococcal strain treated with undiluted, ferritin labelled homologous immunoglobulin The increase in the number of ferritin particles bound to the surface corresponds fairly well to the increase in globulin molecules offered (cf Fig 5) In addition much unlabelled protein is visible on the surface, as could be expected because of incomplete labelling

Binding of serum globulins demonstrated by the indirect staining technique Figs 10 and 11 show a pA pos and a pA neg strain of *S aureus* pretreated with homologous unlabelled rabbit antistaphylococcal serum washed and post treated with ferritin labelled sheep antirabbit globulin On the surface of the pA pos cell (Fig 10) a dense even layer of protein is clearly visible Outside this layer of protein a more uneven, tufty layer of ferritin labelled protein can be seen The pA neg strain (Fig 11) prepared in the same way shows a rather localized antibody adsorption the appearance of which is accentuated by the accumulation of tufts of the ferritin labelled antiglobulins Control preparations of the cells demonstrated in Figs 10 and 11 showed sparse adsorption of the sheep antirabbit glo

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Fig 6 *N gonorrhoeae* (17924) treated with ferritin labelled rabbit antigonococcal globulin Note the sparse adsorption of labelled and unlabelled protein Magnification 90 000 ×

Fig 7 *A pA pos strain of S aureus (4972) treated* with ferritin labelled rabbit antigonococcal globulin The adsorption of protein is similar to that shown with normal rabbit globulin (Fig 2) Magnification 90 000 ×

Fig 8 *N gonorrhoeae* (17924) treated with unlabelled rabbit antigonococcal globulin As compared to Fig 6 a more abundant and more evenly distributed protein layer can be seen Magnification 90 000 ×

Fig 9 A pA neg strain of *S aureus* (E 1369) treated with undiluted ferritin labelled rabbit antistaphylococcal globulin. As compared to Fig 5 the number of ferritin particles bound to the surface has increased Magnification 90 000 ×


10
11
12

bulin. Apparently the cells of the pA pos strain of *S. aureus* did not bind sheep globulin to a higher degree than the pA neg strain did.

Supplementary experiment. The non-immunological affinity of the protein A antigen was also demonstrated by means of another system. Cells from a pA pos and a pA neg strain of *S. aureus* were treated with unlabelled rabbit antiferritin serum, washed and subsequently incubated with a ferritin solution. For control, both strains were incubated with normal rabbit serum instead of antiferritin serum as the first step and also with ferritin alone. Ferritin did not combine directly with staphylococcal cells, and the unlabelled serum could be distinguished on the surfaces of pA pos cells but not on the pA neg cells. Ferritin was not bound to any of the cells pretreated with unlabelled normal serum. In contrast, the pA pos cells pretreated with antiferritin serum showed a strong and uniform binding of ferritin on the outer limitation of the protein already adsorbed to the cells. Fig. 12 shows that the demarcation line between cell surface and protein layer is well pronounced and that ferritin particles form a uniform and constant outer delineation of the cell-globulin-ferritin complex. The pA neg strain did not bind the antiferritin serum.

DISCUSSION

The aim of the present study was to substantiate the difference between immunological and protein A mediated adsorption of rabbit serum globulins to *S. aureus*. To serve this purpose, use of ferritin labelled globulins and electron microscopy was an obvious choice.

Already at an early stage of the development of "immune electron microscopy", *Smith et al.* (21) demonstrated the attachment of ferritin labelled immunoglobulins to cells of *S. aureus*. However, the micrographs published do not permit any detailled evaluation of the degree of binding.

Two electron microscopy studies on the protein A reactivity of *S. aureus* have been published recently. *Nickerson et al.* (14) studied the reactivity between protein A of *S. aureus* Cowan I and human IgG myeloma globulins and *Virgilio et al.* (23) examined the binding of normal human immunoglobulin G to cells of *S. aureus* Cowan III. Both groups of workers found a globulin coating on the entire bacterial surfaces.

The experiments reported here demonstrate convincingly the quantitative difference between immunological and non-immunological adsorption of rabbit serum globulins to the surface of staphylococcal cells. Each cell of a protein A producing (pA pos) bacterial population was completely covered by an even, dense layer of globulin molecules. In contrast, the degree of adsorption in the two different antigen-antibody systems investigated (pA neg *S. aureus*-rabbit antistaphylococcal serum and *N. gonorrhoeae*-rabbit antigonococcal serum) was surprisingly sparse and fortuitous as compared to the results obtained with other antigen-antibody systems by *Shands* (19) and *Swanson et al.* (22). The obvious quantitative difference between specific and non-specific binding is in accordance with the changes in serum globulin concentration found in absorption experiments using the same staphylococcal strains (11). Absorption of homologous antiserum with pA neg strains caused insignificant changes in the concentration of γG globulin, whereas absorption with pA pos strains left only $^{1}/_{10}$ to $^{1}/$[illegible] of the original amount of γG globulin. However, the specific antibodies were removed completely in both cases.

Fig. 10. (Indirect technique, see text). A pA pos strain of *S. aureus* (8341) treated with homologous antiserum. Note the even layer of protein covering the cell and outside this small tufts of ferritin particles. Magnification 90 000 ×.

Fig. 11. (Indirect technique, see text). A pA neg strain of *S. aureus* (E 1369) treated with the same antiserum as the pA pos strain shown in Fig. 10. Note the rather localized antibody adsorption. Magnification 90 000 ×.

Fig. 12. Cells from a pA pos strain of *S. aureus* (8341) treated with unlabelled rabbit antiferritin serum, then washed and incubated with a ferritin solution. Note the strong and uniform binding of ferritin particles. Magnification 90 000 ×.

Protein A antigen seems to reside in the outermost layer of the staphylococcal cell wall, and in the strains studied it was evenly distributed over the whole cell Treatment with trypsin did not alter the structure of the cell wall, whereas the affinity to γG globulins was abolished (11, 14, 23) With the experimental conditions used, localization of other antigenic sites on the bacterial surface was not possible

The sheep globulin employed in the indirect technique did not react with protein A This observation may be explained by the fact that the protein A reactivity with sera of ruminants is limited to the slow γG-globulin component, and the concentration of slow γG globulin is subjected to pronounced individual variation (12)

It is likely that the small tufts of ferritin labelled globulins directly attached to both pA pos and pA neg cells were due to specific staphylococcal antibodies

The binding site for protein A is situated in the Fc fragment of the globulin molecule (1, 2, 5, 6, 13) In an experiment where rabbit antiferritin globulin was bound to the cell surface of pA pos staphylococcal cells prior to exposure to the antigen (ferritin), it was demonstrated that the antibody combining sites are free after the globulin molecule has combined with protein A However, the orientation and shape of the molecules were not demonstrated directly in the present series of experiments

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STUDIES ON THE COMPLEMENT FIXATION TEST WITH *MYCOPLASMA PNEUMONIAE* ANTIGEN

7 Purification of Not Cell Bound Antigen in Broth Culture

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Methods for purifying the not cell bound complement fixing antigen (NCBA) of *M pneumoniae*, present in cellfree filtrates from broth cultures of the organism, have been examined Precipitations with ammonium sulphate (AS) and isopycnic CsCl gradient centrifugation have both proved effective methods by which to separate NCBA from broth medium proteins Using 31 per cent AS, ab 40 per cent of the NCBA measured in native filtrates was regained in the precipitates, the protein content per CF unit of which was ab 5 per cent of that in the native filtrates With increasing salt concentrations the protein figure of the precipitates rose far more steeply than the NCBA yield resulting in an inverse ratio between the yield and the degree of purification The AS precipitation method also facilitated a substantial concentration of NCBA In the CsCl gradient centrifugation NCBA was located in the buoyant density range 1 084 1 105 g/ml indicating a lipid nature of NCBA By a combined application of 31 per cent AS precipitation and CsCl gradient centrifugation, a concentrated preparation of NCBA was produced with a yield on the 30 per cent level and a protein figure below 1 per cent of that in native filtrates

The not cell bound complement fixing antigen (NCBA) of *M pneumoniae* in broth culture has been described in earlier papers referred to in (3 4) NCBA is readily filterable through membrane filters which effectively retain colony forming units In native filtrates NCBA is present in a highly impure state The purpose of the present examination has been to determine methods by which the antigen might be purified, the protein figures and the complement fixing (CF) titres of the materials have been used as parameters The combined use of precipitation with ammonium sulphate (AS) and isopycnic gradient centrifugation employing cesium chloride was found to be effective in attaining a concentration and a separation of NCBA from broth medium proteins The results of the application of these methods are reported

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MATERIALS AND METHODS

The Bård strain of *M pneumoniae* was cultivated in PPLO broth medium *M pneumoniae* cells washed free from NCBA were used as inoculum The development of NCBA in the broth cultures was examined by the direct measurement technique Within 2 days of NCBA being first demonstrable in filtered samples of a culture the whole broth culture was passed through Millipore ® filter discs type GS The native filtrate thus obtained was the starting material of the purification experiments All methods employed in the production of native

filtrates along the above lines are referred to in (3) The CF technique employed is described in (2) After inactivation at 56° C for 30 min, the materials were tested against a diluted anti *M pneumoniae* rabbit hyperimmune serum containing 4 CF units for linear measurements of their CF-titres The CF titre was read as the dilution giving 50 per cent inhibition of haemolysis. For estimation of the protein contents of the materials, two methods were applied 1 the Folin Ciocalteu reagent was used as described and referred to in (2) 2 a modified Biuret method was employed as described in (7) The results of the Biuret tests were read in a Perkin Elmer Spectrophotometer at wavelengths of 310 mμ and 390 mμ and the pro-

filtrate was adjusted to 7 with 1 M Tris maleate buffer pH 8 1 (5) The AS precipitation technique employed was as described in (3) After centrifugation the precipitate was resuspended in veronal buffered saline pH 7 2 ("diluent) and then dialysed at +4°C against several changes of diluent until the addition of a saturated solution of BaCl to a sample of the dialysate no longer resulted in a visible precipitation thus indicating a high degree of freedom from AS The volume of the AS precipitate after dialysis was measured For isopycnic gradient centrifugations, a 60 per cent w/w stock preparation of CsCl "BDH" was used for preparing aqueous solutions with buoyant densities of 1 4 1 3 1 2 and 1 1 g/ml One ml of each solution was deposited in layers in the foregoing sequence in 5 ml cellulose nitrate centrifuge tubes with 1 ml of the material to be purified at the top The tubes were centrifuged in a Spinco ultracentrifuge model L rotor SW 50 at 45 000 rpm for 19 hours The tubes were harvested from the bottom by puncture 18 fractions of equal volume being collected by means of a Buchler polystaltic pump The buoyant density of individual fractions was determined by direct weighing in tared 0 1 ml micropipettes

EXPERIMENTAL

The effect of the AS concentration upon the yield and degree of purification of NCBA was examined by precipitation experiments with three different salt concentrations these are defined as follows

31% AS	to 100 ml filtrate was added 18 13 g AS
36% AS	to 100 ml filtrate was added 21 75 g AS
47% AS	to 100 ml filtrate was added 29 00 g AS

% AS corresponds to per cent saturation at 25° C (6

Each concentration of AS was added to a separate volume of 24 ml filtrate, the amount of AS to be used was calculated from the total volume obtained after addition of the amount of 1M Tris maleate buffer needed to bring the pH to 7 0 The precipitations were performed as described in "Methods" The precipitates were resuspended in 1 5 ml diluent and the actual volumes of the dialysed precipitates gauged at the end of the dialysis The CF titres of the three precipitates and that of the native filtrate were measured in the same CF set-up Two different native filtrates, produced in separate cultivation experiments, were precipitated with AS as described All measurements of the protein figures of the filtrase and the AS precipitates in Exp 1 were performed by the Biuret method in Exp 2 the Folin test was used throughout A separate preparation of 31 per cent AS precipitate was further purified by CsCl gradient centrifugation One ml AS precipitate was deposited on the top of each of two identical tubes as described in "Methods" After centrifugation and fractionation of the gradients, the CF titres of the fractions and that of the 31 per cent AS precipitate were measured in the same CF set up. Attempts to estimate the protein figures of the fractions from Gradient 1 by the Folin test were partially unsuccessful as precipitates were formed following addition of the Folin-Ciocalteu reagent In fractions rich in proteins, the blue colour formed was bound to the precipitate The protein figures of the fractions from Gradient 2 were estimated by the Biuret method

RESULTS

The two separate filtrates employed in the AS precipitation experiments had CF-titres of 12 and 10, and their protein figures were 22 000 and 14,750 μg/ml respectively Table 1 shows the yields and the protein figures of the resulting precipitates The yields are given as the number of CF units (CF titre × volume) measured in the dialysed precipitates expressed as a percentage of the number measured in 24 ml native filtrate The protein figures in μg/ml per CF unit were calculated by dividing the protein figures of the precipitates in μg/ml by their CF titres The values found in the native filtrates are also given at the foot of the table The table shows that by using 31 per cent AS 42/35 per cent of the NCBA in the native filtrate was recovered in the precipitate, the protein figure of which in μg/ml per CF unit was

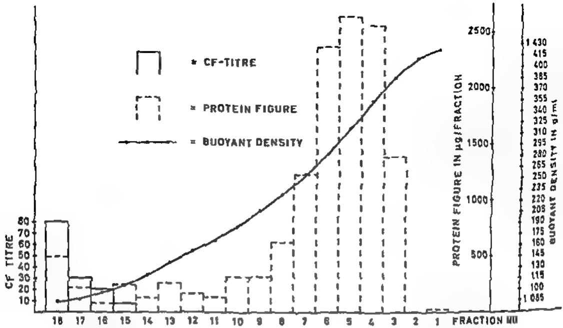

Fig 1 *Purification of a 31 per cent ammonium sulphate precipitate (see text) of not cell bound complement fixing antigen (NCBA) of Mycoplasma pneumoniae by means of isopycnic CsCl gradient centrifugation The CF titres protein figures and buoyant densities of individual fractions are shown*

4/7 per cent of that in the native filtrate With increasing salt concentration the protein figure of the precipitates rose far more steeply than the NCBA yield

CF examination of the fractions from the CsCl gradient centrifugations yielded very similar results in both gradients, the highest titre being found in the top fraction (fr 18), this fraction had a higher CF titre than the 31 per cent AS precipitate to be purified The following three fractions (17–15) showed decreasing titres, and no NCBA could be traced below fraction 15 The bottom fractions, 6–1, displayed an anticomplementary effect when tested in diluent The CF results of Gradient 2 are shown in Fig 1, which also gives the protein figures and the buoyant density values of the fractions The NCBA containing fractions had a buoyant density of from 1 084 g/ml in fr 18 to 1 105 g/ml in fr 15 Protein was measured in all fractions (except fr 2), but the distribution of proteins was characterized by a marked concentration in the fractions 7–3, corresponding to

TABLE 1 *Precipitation with Ammonium Sulphate (AS) of Not Cell Bound Complement Fixing Antigen of* Mycoplasma pneumoniae *from Native Broth Culture Filtrates—Effect of the AS Concentration upon the Yield and the Protein Content of the Precipitates The results of Two Experiments Are Shown*

% AS	No of CF units precipitated		Protein content of precipitates	
	abs fig	in % of filtrate fig	in µg/ml per CF unit	in % of filtrate f g
31	120/84	42/35	73/104	4/7
36	191/116	66/48	234/386	13/26
47	234/221	81/92	580/621	32/42
Native filtrate	288/240		1833/1475	

TABLE 2 *Purification of a 31 Per Cent Ammonium Sulphate (AS) Precipitate (See Text) of Not Cell-Bound Complement Fixing Antigen (NCBA) of* Mycoplasma pneumoniae *by Means of Isopycnic CsCl Gradient Centrifugation The yield and the Protein Figure of the NCBA-Containing Fractions Compared with That of the AS Precipitate Are shown*

	No of CF units	Total protein in µg	Protein in µg/ml per CF unit
1 ml 31 % AS precipitate	48	12,850	268
Fractions 18–15*	39	1,023	26
Fractions 10–1	39	13 066	

* buoyant density range 1 081 1 105 g/ml

TABLE 3 *pH 5 3 Treatment of a 31 Per Cent Ammonium Sulphate (AS) Precipitate of Not Cell Bound Complement Fixing Antigen of* Mycoplasma pneumoniae *(See Text) The Effect upon the CF-Titre and the Protein Figure in Two Experiments Are Shown*

	CF-titre	Protein in µg/ml	Protein in µg/ml per CF unit
31 % AS precipitate untreated	140/128	12 050/9,270	86/72
31 % AS precipitate treated at pH 5 3	100/64	8 850/8,000	89/125

the buoyant density range 1 253–1 382 g/ml Table 2 shows the NCBA yield and the protein figure of the combined fractions 18–15 Out of 48 CF-units measured in the 31 per cent AS precipitate, 39 CF-units (81 per cent) were recovered in the combined top fractions, the protein figure of which in µg/ml per CF unit was $^1/_{10}$ of that in the AS precipitate

Purification of AS precipitate of NCBA at pH 5.3 This method has been applied to 31 per cent AS precipitate by a procedure described in (4), it consists of removal by centrifugation of material which is easily precipitable at pH 5 3 The procedure removes the slight bluish white opaqueness present in the untreated 31 per cent AS precipitate (3) The procedure was found essential as a pre-treatment when 31 per cent AS precipitate was used as antigen in immune electron microscopy of NCBA (4) The effect upon the CF titre and protein figure is illustrated in Table 3 which shows that the procedure causes a stronger reduction of the NCBA content than of the protein content of the 31 per cent AS precipitate, resulting in an increase in the protein figure in µg/ml per CF unit

DISCUSSION

In the present work, both AS precipitations and isopycnic CsCl gradient centrifugation have proved effective in separating NCBA of *M pneumoniae* from broth medium proteins The problem of separating NCBA from non-protein medium constituents e g broth medium lipids, has not been investigated The precipitability of NCBA of *M pneumoniae* with AS has been reported earlier (1) and has been examined in more detail in the present work With this method, an inverse ratio between the yield and the degree of purification has been demonstrated (Table 1) However even the AS precipitates with yields on the 80–90 per cent level showed significantly lowered protein figures From the relatively high yields of NCBA obtained with low salt concentrations (31 per cent AS), NCBA of

M pneumoniae can be characterized as easily precipitable with AS The AS precipitation method facilitates a substantial concentration of the antigen In the CsCl gradient centrifugation, a further purification of the NCBA in the AS precipitates from broth medium proteins was achieved, and in the top fraction a concentration occured as well The distribution of antigen between the four top fractions found in the present experiments may, at least partially, have been affected by the technique employed for harvesting the gradient Antigen was found in the buoyant density range 1 084–1 105 g/ml, indicating a lipid nature of NCBA of *M pneumoniae* By the combined application of 31 per cent AS precipitation and CsCl gradient centrifugation to native filtrates from broth cultures of *M pneumoniae,* a concentrated preparation of NCBA was produced with a yield on the 30 per cent level and a protein figure below 1 per cent of that in the native filtrate

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FURTHER OBSERVATIONS ON THE ULTRASTRUCTURE OF *TREPONEMA PALLIDUM* NICHOLS

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Flagella isolated from *Treponema pallidum* Nichols after treatment with the detergents Teepol and sodium deoxycholate, and with the enzyme *Myxobacter* AL 1 protease 1, were studied in the electron microscope after negative staining The flagella were found to consist of 1) a sheathed shaft, 2) a hook with a honey combed substructure, 3) a narrow collar with a smooth appearance, and 4) a basal knob which seemed to consist of a single disc Comparison was made with descriptions of flagella isolated from other spirochetes, and with flagella isolated from gram negative bacteria Treatment of *Treponema pallidum* Nichols with sodium deoxycholate or with the protease 1 (AL–1 enzyme) revealed two bundles of intracytoplasmic microtubules, one bundle at each end of the organism Each bundle consisted of 6–8 microtubules and each tubule had a diameter of 7–7 5 nm The possible taxonomic significance of the presence of microtubules in some species of *Treponema* is discussed

In an earlier paper on the ultrastructure of *Treponema pallidum* Nichols (9) the number and mode of attachment of the intracellular filaments were described The substructure of these filaments was found to be similar to that of bacterial flagella The results of further studies on these filaments or flagella liberated by disruption of the organisms after chemical treatments and enzymatic digestion are presented in the present paper The structures observed at the insertion ends of the filaments are compared with those of this region of bacterial flagella (1, 4, 13), with the structures of the corresponding parts of flagella isolated from *Treponema* Reiter (7) and with those of the insertion region of axial filaments isolated from some species of *Leptospira* (3, 11)

The presence in *T pallidum* Nichols of two bundles of intracytoplasmic microtubules resembling those observed in *T* Reiter (7) is also reported

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MATERIALS AND METHODS

T pallidum Nichols was maintained by intra testicular passage in rabbits, and the organisms were purified as described by *Jepsen et al* (9)

The purified treponemes were treated with 0 2 per cent Teepol or 1 per cent sodium deoxycholate in redistilled water, or with a solution of AL 1 enzyme*) containing 100 μg/ml of the enzyme and 5×10^{-4} M of EDTA in $2\,5 \times 10^{-3}$ M TRIS buf

*) The purified enzyme *Myxobacter* AL–1 protease 1 was kindly provided by Professor *R S Wolfe*, Department of Microbiology, University of Illinois, Urbana, Ill, U S A

M. pneumoniae can be characterized as easily precipitable with AS. The AS precipitation method facilitates a substantial concentration of the antigen. In the CsCl gradient centrifugation, a further purification of the NCBA in the AS precipitates from broth medium proteins was achieved, and in the top fraction a concentration occured as well. The distribution of antigen between the four top fractions found in the present experiments may, at least partially, have been affected by the technique employed for harvesting the gradient. Antigen was found in the buoyant density range 1.084–1.105 g/ml, indicating a lipid nature of NCBA of *M. pneumoniae*. By the combined application of 31 per cent AS precipitation and CsCl gradient centrifugation to native filtrates from broth cultures of *M. pneumoniae*, a concentrated preparation of NCBA was produced with a yield on the 30 per cent level and a protein figure below 1 per cent of that in the native filtrate.

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fer pH 9 0 (4) and negatively stained The procedures used are described in detail in a previous paper (7) The stain used was 1 per cent ammonium molybdate adjusted to pH 7 with NH_4OH

Electron microscopy was carried out on a Philips EM 200 electron microscope at primary magnifications of 9000 × and 16 000 × Negatives were obtained on Kodak Fine Grain Release Positive Film Type 5302 and were photographically enlarged as desired Approximately 300 recordings were studied in these experiments

RESULTS

Treatment of *T pallidum* Nichols with 0 2 per cent Teepol for about 2 minutes disturbed the regular, wavy appearance of the cells The organisms appeared straighter than well preserved organisms from control preparations

Teepol treatment tears the flagella loose from the cells and the insertion ends of the flagella are seen lying free on the supporting film of the grid a little away from the tip of the treponeme Often the shaft of the flagella may still wind around the cytoplasmic body of the treponeme (Fig 1) The wavelength of the flagella appears to be the same as that of flagella in untreated or mechanically damaged organisms (Figs 1, 2 & 3)

All illustrations show material from *Treponema pallidum* Nichols negatively stained with 1 per cent ammonium molybdate Unless otherwise stated the bar on each micrograph represents 100 nm

Fig 1 Part of an organism treated on the grid with Teepol for 2 min Flagella (F) wind around the cytoplasmic body of the cell though the insertion ends (arrow) are torn out of the cytoplasm 90 000 ×

Fig 2 Flagellum liberated after treatment as for Fig 1 The wavelength appears to be the same as for flagella from untreated organisms 90 000 ×

Fig 3 Flagellum liberated after treatment as for Fig 1 The hook (H) starts where the substructure of the shaft (S) changes abruptly (arrow) B denotes the basal knob 175 000 ×

Fig 4 Flagella liberated after treatment as for Fig 1 The narrow collar (C) connects the basal knob (B) to the hook (H) The substructure of the collar differs both from that of the hook which is honey-combed and that of the shaft (S) 320 000 ×

The flagellum consists of a shaft proper a hook and a basal knob (Figs 2 3, 4 & 5) The shaft has a diameter of about 17 nm The substructure of the hook is honeycombed (Fig 4) and the width is about 17 nm (Figs 3, 4 & 5)

A narrow collar connects the hook to the basal knob (Figs 3 & 4) The diameter of this collar is about 9 nm and the length about 13 nm The substructure of the collar is different from both that of the hook and the shaft (Figs 3 & 4) On a few micrographs a ring or plate can be detected on the collar quite close to the basal knob (Fig 5)

The diameter of the basal knob is difficult to measure because of attached remnants of membranes It seems however, to be about 30–35 nm (Figs 3, 4 & 5)

Most of the flagella are still inserted into the cells after treatment with 1 per cent sodium deoxycholate Prolonging the treatment as much as to 20 hours does not liberate a greater number of flagella The wavy appearance of the organisms is somewhat distorted by the treatment However, the pointed ends of the cells are still evident, and the flagella are inserted subterminally Thin fibrils are observed in the interior all along the bodies of the treated cells (6)

Treatment with *Myxobacter* AL–1 protease 1 rapidly disrupts the organisms After 30 seconds, flagella thin fibrils and a few pieces of membrane originating either from the cell wall or the cytoplasmic membrane are all that remain of the treponemes (Fig 7)

The shaft of the flagellum appears to be covered by a sheath which can be removed by repeated washing of the flagella with redistilled water after AL 1 enzyme treatment The naked flagellum has a diameter of about 10 nm (Fig 8)

The basal knobs of the flagella are usually seen to be connected with pieces of membrane after the treatment of the treponemes with AL–1 enzyme (Fig 9), even when the treatment is prolonged to 60 minutes Ring shaped structures surround some of the basal knobs (Fig 10)

On closer inspection of several micrographs


5
H
C
B
6
M
F
7
M
F
8
F
F

some negative staining material can be seen to have penetrated into the central region of the thin fibrils These are, therefore, most probably hollow structures thin tubes or microtubules (Figs 7 & 11) Other micrographs show that each organism contains two bundles of microtubules one bundle originating or ending close to the insertion points of the flagella at either end of the organism (Fig 12) In the mid region of the organism the microtubules of each bundle overlap or interdigitate (Figs 13 & 14) As many as 16 microtubules have been found in this region Each bundle of microtubules consists of 6–8 individual tubules with a diameter of about 7–7 5 nm (Figs 7, 11 & 12)

DISCUSSION

T pallidum Nichols seems to be rather resistant to disruption with the detergents Teepol and sodium deoxycholate While treatment with 0 2 per cent Teepol for 30 seconds removed most of the flagella from *Treponema* Reiter (7) *T pallidum* Nichols had to be treated for about 2 minutes to obtain the same effect Flagella could be isolated from *Treponema* Reiter after treatment of suspended organisms with 1 per cent sodium deoxycholate for 30 minutes (7) The same treatment prolonged to 20 hours did not

Fig 5 Flagella liberated after treatment as for Fig 1 A ring or plate (arrow) is seen on the collar (C) close to the basal knob (B) 160 000 ×

Fig 6 Part of an organism treated in suspension with sodium deoxycholate A bundle of microtubules (M) is seen in the interior of the cell. Flagella (F) are also present The pointed end of the cell is still evident after the treatment 90 000 ×

Fig 7 Remnants of an organism treated on the grid for 30 seconds with AL 1 protease I Only flagella (F) a bundle of microtubules (M) and membranous debris (arrows) are left 160 000 ×

Fig 8 Fragments of flagella which have lost their sheaths after repeated washings with redistilled water (arrows) Some flagella (F) still retain their sheaths The flagella were liberated by treatment in suspension with AL-1 protease I 90 000 ×

liberate the flagella from *T pallidum* Nichols It is not known whether this greater resistance to detergent treatment is a species property, or whether it is caused by a protective effect of rabbit tissue debris still present in the *T pallidum* suspension

The observations on the substructure as well as on the width of sheathed and unsheathed flagella are all in accordance with observations on isolated flagella of *Treponema* Reiter (7) and of *Leptospira* spp (11, 3), and on flagella isolated from gram negative bacteria (1, 4, 13)

The narrow collar that connects the basal disc and the hook has also been observed on filaments isolated from *Spirochaeta stenostrepta* (6) and on flagella isolated from *Treponema* Reiter (7) It is also seen on a micrograph of a filament isolated from *Spirochaeta litoralis* (5)

The basal knob of flagella isolated from *T pallidum* Nichols has been very difficult to resolve Studies of micrographs where the insertion end of the flagella is not too obscured by remnants of membranes suggest that the basal knob consists of one single disc, resembling that of *Treponema* Reiter (7)

The ring like structures surrounding the basal knobs of membrane attached flagella after treatment with *Myxobacter* AL-1 protease I are also seen after treatment of *Treponema* Reiter with the same enzyme (7)

The small ring on the narrow collar which is seen on a few flagella only, is probably pieces of adhering membrane or side views of the ring shaped structures seen in Fig 10

The microtubules with a diameter of about 7 nm have hitherto been observed in treponemes only Evidence for a connection between the microtubules and the flagella has not been found though their end points are close together (Fig 12)

In thin sections of *T pallidum* Nichols the bundle of microtubules can be seen situated in the cytoplasm close to the inner leaflet of the cytoplasmic membrane and right underneath the bundle of flagella (9 (Fig 16), 10), as has been demonstrated for *Treponema* Reiter (7)

H
C
B
9
10
11
12

1

14

Fig 13 The mid region of a cell treated as for Fig 12 The bundle of microtubules consists of about 10 individual tubules 160,000 ×

Fig 14 Mid region of the same organism as shown in Fig 7 Two bundles of microtubules are present They tend to come apart, probably as a result of tearing during preparation 160,000 ×

In Fig 7, a part close to an end of the same organism is shown There, only one bundle of microtubules consisting of 7–8 individual tubules is present.

Fig 9 Flagella liberated after treatment on the grid for 4 min with AL-1 protease 1 Pieces of membrane are seen to adhere to the basal knobs (B) The narrow collars (C) connect the basal discs to the hooks (H) 160 000 ×

Fig 10 Flagella liberated after treatment as for Fig 7 Note the ring-shaped structure surrounding the basal knobs (arrows) 160,000 ×

Fig 11 Microtubules from an organism treated as for Fig 7 Note that electron dense stain is present in the lumen of the microtubules (arrows) Several of the microtubules appear broken 160,000 ×

Fig 12 End of a cell treated on the grid for 4 min with AL-1 protease 1 The ends of the microtubules are seen close to the insertion points of the flagella (arrow) 160 000 ×

At present microtubules have been demonstrated in *T. cuniculi* (8), *T pallidum* (10 and this study), *T pertenue* (12) and *Treponema* Reiter (7) In this laboratory, work is in progress to investigate whether these microtubules are unique for the genus *Treponema* If so, they can probably be used as a criterion for the classification of this genus Until now treatment of some *Leptospira* spp with sodium deoxycholate or with *Myxobacter* AL-1 protease 1 has not shown any evidence of microtubules (3), nor has treatment with AL-1 enzyme of gram-negative bacteria revealed microtubules (4)

In *Treponemataceae* the flagella-like filaments situated between the cell wall and the cytoplasmic membrane have been termed in-

ternal fibrils or filaments, or axial fibrils or filaments The author would like to propose that these should be designated flagella, for the following reasons

1) They have the same dimension and the same ultrastructure as bacterial flagella

2) They are made up of the same main constituents as bacterial flagella, as preliminary chemical analysis suggests (2, 11)

3) They are not axially located, but wind around the cytoplasmic bodies of the organisms

4) The term internal fibrils or filaments can be confused with the term intracytoplasmic filaments which some authors use for the microtubules observed in the cytoplasm of several species of *Treponema*

In previous papers (7, 9) it was proposed that the filaments of *Treponema* Reiter and *T pallidum* Nichols should be termed endoflagella However, there seems to be some reluctance among bacteriologists to use the prefix 'endo' for something which does not have an intracytoplasmic location Consequently, in order to avoid further confusion it is proposed to use the term flagella for the internal fibrils of the treponemes

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INTRAVASCULAR CLEARANCE OF ENDOTOXIN IN WARFARIN—TREATED RABBITS

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The primary elimination from circulating blood of intravenously administered endotoxin and the primary distribution of the phagocytised endotoxin have been compared in warfarin pretreated and untreated rabbits The results show that the elimination rate of endotoxin is significantly higher in warfarin treated rabbits than in untreated The difference is, however, small, and does not reflect a significant difference in the organ distribution although the amounts of endotoxin in the organs are somewhat higher in the warfarin-treated animals Twenty min after injection about 75 per cent of the injected endotoxin has been eliminated from circulation, and about 50 per cent of the injected dose is located to the liver The highest amount of endotoxin per g tissue is, however, found in the lungs Twenty min after injection the same percentage, about 90, of circulating endotoxin was in both groups located to the plasma phase The fact that the clearance rate of endotoxin in warfarin treated rabbits is higher than in untreated rabbits is thought to be due to the existence of a competitive phagocytosis of endotoxin and fibrin clots and not to a changed *endotoxinplatelet* interaction

Intravenously administered endotoxin disturbs the dynamic equilibrium of the coagulation system and gives an immediate hypercoagulability followed by an accelerated, disseminated intravascular coagulation leading to hypocoagulability and a consumption coagulopathy (Verbrauchskoagulopathie) (25 26, 28, 42) The trigger mechanism is still uncertain (8), but is probably an endotoxin-platelet interaction followed by release of platelet factor 3 (7, 24, 26 28, 34)

The changes in coagulability parallel a decrease in the clearance function of the reticuloendothelial system (R.E.S.) To some extent this may be due to an overload of lipids and intermediary coagulation products (25, 30) A competitive phagocytosis of fibrin clots and endotoxin has also been suggested The intravascular coagulation is further accelerated after pretreatment with agents which block the R.E.S. function (25, 27)

The increased intravascular coagulation is inhibited by pretreatment with heparin or oral anticoagulants (11, 24, 29 36) It is reasonable to suggest that the major factor is their anticoagulant effect (28, 33 36), but they may also have a more direct influence on the clearance function of the R.E.S. (1 10 32) The published results are, however controversial, and the mechanism is not clear (10, 23, 42)

The present study is concerned with how pretreatment with the anticoagulant warfarin sodium (warfarin) influences the immediate

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vascular clearance of intravenously administered endotoxin, and its distribution in various organs in rabbits

MATERIALS AND METHODS

Animals Twenty 'White Land' rabbits, caged, fed and chosen as described in an earlier paper (15), were divided into two groups, A and B, each comprising 5 male and 5 female rabbits The mean weights of the rabbits were approximately the same in the two groups, 4 0 kg (Variation 3 6–4 6) and 3 9 (Variation 3 2–4 6), respectively The age of all rabbits was 6 months

Anticoagulant treatment with warfarin, and Thrombotest determination of the coagulation activity in per cent (TT %), were carried out as described earlier (16) Group A was chosen at random for anticoagulant treatment

Endotoxin Bacto Lipopolysaccharide B (L P) from *S typhi 0901* (Difco) (control No 511771), stored at 4° C, was used

⁵¹Cr-labelling of endotoxin Chromium-51 was supplied as chromic chloride in isotonic, sterilized solution with pH 3 1 and specific activity 226 78 mC/mg Cr (The Radiochemical Centre, Amersham/ Institutt for Atomenergi, Kjeller, Norway) Except for a reference standard, the solution was mixed with a solution of endotoxin according to Braude et al (4), so that a total volume of 10 0 ml, pH 7 2 contained 5 mg endotoxin/ml The mixture was incubated at 37° C for 28 hrs and agitated continuously It was then dialyzed at 4° C against distilled water, using Dialyzer Tubing No 4465 A2 (Arthur H Thomas Comp Philadelphia), until the water outside the bag showed only a trace of radioactivity In the dialyzed mixture 1 mg endotoxin was found to bind 2 34 mcg Cr The endotoxin was still able to elicit antibody formation in rabbits The mixture, except a subsample for use as a reference standard, was diluted with 0 9 per cent NaCl to a concentration of 0 25 mg endotoxin/ml and pH 7 0 The rabbits were given 0 15 mg endotoxin/kg body weight (b w), by injection through the left ear vein over a period of 30 seconds

Bacterial agglutination test was performed as previously described (17)

Determinations of the volume of packed red cells (V P C) and the *erythrocyte sedimentation rate* (E S R.) were performed according to the techniques described by Wintrobe (41)

Blood samples, 1 ml volumes were obtained from the right marginal ear vein at 1 min intervals in the period 4½ 16½ min after the endotoxin injection The samples were transferred immediately to flat bottomed tubes containing 3 0 ml 3 8 per cent (w/v) sodium citrate Blood samples collected when the rabbits were killed were examined for V P C and E S R, and for radioactivity of whole blood, plasma and blood cells Plasma and blood cells were separated after preparation and centrifugation, as for the determination of V P C

Autopsy and organ specimens Exactly 20 min after the endotoxin injection the rabbits were killed and bled out A macroscopic histological examination was performed, and lungs, heart, liver, spleen and kidneys were removed and weighed (Mettler scales 1 div = 0 1 mg) The relative weight of the various organs *i e* organ weight/animal weight was calculated A minimum of 10 representative specimens was taken from different parts of each organ, transferred to a flat bottomed tube and digested with conc HNO_3, the total volume being 3 8 ml

Radioassay was carried out with a scintillation counter 6006 IDL equipped with a well type scintillator (NaI(TA) crystal 13/4"×2), and PW

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toxin content was calculated according o the radioassay of the reference standard

Vascular clearance (disappearance) curve The intravascular endotoxin decrease was plotted as a function of time on semilogarithmic paper, and the best fitting curve drawn The curve interval 4½–14½ min after the endotoxin injection was resolved in two components representing the 5 first and the 5 last endotoxin concentrations, respectively An approximation was made to linearize the equations for the two components, and their regression coefficients were calculated The absolute values of the regression coefficient correspond to the phagocytic index, K The corresponding intravascular clearance half time, T/2, for the endotoxin was calculated for each component from the formula

$$T/2 = \frac{\log_{10}2}{K} = \frac{0\,301}{K}$$

Statistics The term mean refers to the arithmetic mean The standard deviation and the standard error of the mean (S E) were calculated by common statistical methods Analysis of variance (two way classification) and regression analysis by the least squares method were performed according to Sverdrup (39) The significance of the difference between two means was tested by the t test A 5 per cent (0 05) level of significance was used throughout

EXPERIMENTS AND RESULTS

A comparison was made between warfarin treated (group A) and untreated (group B) rabbits with regard to intravascular clearance

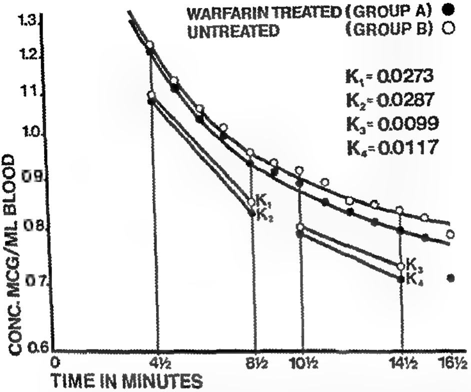

Fig 1 The clearance curve for intravenously administered endotoxin in warfarin treated (group A) and untreated (group B) rabbits for the period 4½–16½ min after injection The straight lines represent the linearized clearance curves for the periods 4½–8½ and 10½ 14½ min respectively The absolute values of the regression coefficients for the lines K_1 K_4 correspond to the phagocytic indices

and to the distribution in organs of intravenously administered endotoxin

The rabbits of group A were treated daily with warfarin for 4 days prior to examination On the day of examination the mean TT per cent of this group was 8 (Variation 6–11) No hemorrhages or other complications related to the anticoagulant treatment were observed during treatment or on autopsy The values of the TT per cent for group B were all over 70, and the mean value was approximately 100

The 0-antibody titre against *S typhi 0901* was below 1 16 in the serum of each rabbit The V P C and E S R were within normal range in all rabbits The mean V P C for the two groups was 43 3 (Variation 39–50) and 41 8 (Variation 38–48) ml/100 ml respectively, and the mean E S R 2 mm/hr with variations 1–5, and 1–3 mm/hr, respectively

The Intravascular Endotoxin Clearance

For each group the mean and S E of the endotoxin concentrations in whole blood at 1 min intervals from 4½ to 16½ min after injection, are given in Table 1 The results for one rabbit of group A were excluded because exactly timed sampling was not obtained The results are visualised by the multiexponential curves in Fig 1

Throughout the period of examination the individual variations were of the same magnitude (Tab 1) within the two groups, the variations being fairly constant for all intervals The mean concentration 16½ min after injection is in both groups definitely lower

than expected (Fig 1) This is, no doubt, due to a technical error

TABLE 1 *The Mean and S E of Endotoxin Content in Peripheral Blood of Warfarin Treated (A) and Untreated (B) Rabbits at 1 Min Intervals between 4½ and 16½ Min after injection*

Minutes after inject	Endotoxin (mcg ^{3}/ml)			
	Warfarin treated		Untreated	
	Mean	S E	Mean	S E
4½	1226	61	1235	59
5½	1110	57	1129	60
6½	1037	54	1063	54
7½	975	50	1010	49
8½	932	48	956	53
9½	911	58	931	51
10½	886	57	913	53
11½	848	49	883	52
12½	832	53	856	47
13½	810	52	843	45
14½	794	51	833	49
15½	779	52	818	46
16½	710	36	785	43

For all intervals the mean endotoxin concentration is lower in the warfarin treated (group A) than in the untreated (group B) rabbits Variance analysis (two way classification) shows a significant difference ($0\,01 < P < 0\,05$) between the two groups, although the t test shows no significant difference between any of the corresponding endotoxin concentrations of the two groups The results (Table 1 and Fig 1) indicate a tendency to a relative *increase of the difference* with increase in time, but this tendency is not significant

For the two periods, 4½–8½ and 10½–14½ min, each representing five endotoxin concentrations, approximations were made to linearize the equations of the two components of each clearance curve as these represented nearly straight lines (Fig 1) Based on this approximation, the endotoxin clearance rate as expressed by the phagocytic index, K, and the half time, T/2 was calculated and the results are given in Table 2 The individual clearance rates vary relatively little and the variations are about the same in both groups The mean K value for both periods is higher in the warfarin treated than in the untreated rabbits, but analysis by the t test shows no significant difference However, in each of the two groups the clearance rate is significantly higher for the first period than for the second

The findings thus indicate that, in the period of examination, intravenously administered endotoxin disappeared from circulation at a somewhat higher rate in warfarin treated than in untreated rabbits The difference between the two clearance curves is not great, but is statistically significant

The Distribution of Endotoxin in Organs

The mean and S E of the absolute and of the relative weight of the various organs of warfarin treated (group A) and untreated (group B) rabbits are given in Table 3 The

TABLE 2 *The Clearance of Endotoxin from Blood in Warfarin Treated (Group A) and Untreated (Group B) Rabbits Expressed by the Mean Phagocytic Index (K), and the Half Clearance Time (T/2) during the Periods 4½–8½ Min and 10½–14½ Min after Injection*

Group	Period 4½ 8½ min			Period 10½ 14½ min		
	K 10²		T/2(min)	K 10		T/2(min)
	Mean	S E	Mean	Mean	S E	Mean
A	2 87	0 20	10 5	1 17	0 21	25 7
B	2.73	0 14	11 0	0 99	0 13	30 4

TABLE 3 *The Mean and S.E. of the Absolute and the Relative Weights of Various Organs of Warfarin Treated (Group A) and Untreated (Group B) Rabbits, respectively, at Autopsy, i.e. 20 Min after Endotoxin Injection*

Organ	Group	Absolute Weight (g)		Relative Weight (g/kg b.w.)	
		Mean	S.E.	Mean	S.E.
Liver	A	95.26	8.04	23.80	1.66
	B	94.92	4.99	24.10	0.84
Spleen	A	0.94	0.08	0.24	0.02
	B	1.07	0.10	0.27	0.02
Kidneys	A	19.51	0.87	4.92	0.18
	B	20.21	0.93	5.17	0.23
Lungs	A	13.41	0.59	3.39	0.17
	B	12.08	0.44	3.08	0.09
Heart	A	7.63	0.31	1.98	0.09
	B	8.00	0.35	2.04	0.06

organ weights were recorded at autopsy, i.e. 20 min after endotoxin injection. No significant difference was found between any of the corresponding weights of the organs from the two groups. The difference in absolute and especially in relative weights of the same organ from different rabbits is small, and about the same within each group.

Distribution of endotoxin among liver, spleen, kidneys, lungs and heart at 20 min after injection is shown in Table 4. The endotoxin content is presented as total per organ, as amount per g organ weight, and as per cent of the injected endotoxin dose. The variation between individual animals within each group is small, and for the same organ the mean values obtained by the three methods have about the same relative variation. The t-test showed no significant difference between the mean values for the two groups, irrespective of the method used for calculation, except for the endotoxin content per g spleen tissue ($0.02 < P < 0.05$). Nor did multiple comparison show any significant difference between the organ contents of the two groups. Nevertheless, although no signifi-

TABLE 4 *The Mean and S.E. of the Endotoxin (L.P.) Content in [illegible] n Treated (Group A) and Untreated (Group B) Rabbits [illegible] Endotoxin Injection. The Endotoxin Content is presented as [illegible] g Organ Weight and as per cent of the I[illegible]*

Organ	Group	Total L.P. mcg		L.P./g Organ mcg/g		L.P./Inj. L.P. %	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Liver	A	343.1	18.8	3.72	0.20	55.74	1.79
	B	321.4	14.8	3.44	0.17	54.72	1.72
Spleen	A	6.3	0.8	6.64	0.52	1.02	0.12
	B	5.3	0.9	4.87	0.56	0.88	0.12
Kidneys	A	3.9	0.3	0.20	0.01	0.64	0.04
	B	3.8	0.2	0.19	0.01	0.65	0.04
Lungs	A	106.6	7.6	7.89	0.27	17.55	1.42
	B	102.4	6.6	8.55	0.52	17.48	0.95
Heart	A	0.8	0.1	0.10	0.01	0.13	0.01
	B	0.9	0.1	0.12	0.01	0.16	0.02

cant difference was found between the groups compared, the amount of endotoxin in the organs, both the total amount and the percentage of the injected amount, is somewhat higher in the warfarin-treated group than in the untreated one. This finding supports the previous finding of a somewhat higher clearance rate in the warfarin-treated rabbits.

It should be noted (Table 4) that 20 min after injection about 75 per cent of the total injected dose is located in the five organs examined, the definitely highest amount, more than 50 per cent of the injected dose, being in the liver and only 1 per cent in the spleen. Noteworthy is also the high percentage of endotoxin in the lungs, and the fact that this organ has the highest content of endotoxin per g tissue.

TABLE 5 *The Endotoxin Content 20 Min After Injection in Whole Blood and the Percentage Located to the Plasma Phase of the Blood in Warfarin Treated (Group A) and Untreated (Group B) Rabbits*

Group	Endotoxin Content			
	Whole Blood (mcg ^{3}/ml)		Plasma Phase (%)	
	Mean	S.E.	Mean	S.E.
A	838	61	97.21	0.61
B	1184	73	96.17	0.26

Endotoxin in Peripheral Blood

The distribution of endotoxin between formed blood cells (elements) and plasma was examined in blood collected 20 min after intravenous administration of endotoxin.

The amounts of endotoxin located to formed elements and to the plasma phase were recorded separately. The percentage of endotoxin in whole blood located to the plasma phase was calculated with regard to the individual V.P.C. The mean percentage value and S.E. for each group is presented in Table 5. The variation between individual animals is small in both groups, the t-test showing no significant difference between the mean values for the two groups.

The mean endotoxin concentration in whole blood at autopsy, i.e. 20 min after injection, is for both groups somewhat higher than that determined 16½ min after injection in connection with the clearance study. This comparison is, however, of limited interest as the methods used for determination and the sampling technique were quite different. It should, however, be noted that the mean concentration obtained 20 min after injection is significantly lower in the warfarin treated rabbits ($P<0.01$). This finding is in accordance with the difference obtained between the two groups in the clearance study.

DISCUSSION

The hypercoagulability which is the immediate result of the intravenous administration of endotoxin is followed by a disseminated intravascular coagulation (13, 25, 28, 40, 42). In the present work the influence of warfarin on the initial or primary elimination from the blood stream and the primary organ distribution of injected endotoxin have been examined. In the warfarin-treated rabbits the coagulation activity was kept at a level which corresponds to the range desired for effective anticoagulant treatment in man (16).

The present observations show that the primary elimination of endotoxin in both warfarin-treated and untreated rabbits follows a multiexponential function (Fig. 1) as noted for untreated animals by previous workers (22). The slopes of the clearance curves, and thus the exact phagocytic rates, are therefore difficult to calculate. The present results, however, show a significant difference between the clearance curves for the two groups. As it seems improbable that the start concentration is different in the two groups it is reasonable to conclude that the difference is due to a somewhat higher clearance rate in the warfarin treated rabbits, although a significant difference between the two curves with regard to time was not

obtained The clearance curves themselves (Fig 1), the concentrations in blood at autopsy and the amount of endotoxin in the organs support this conclusion Clearance curves for the period immediately following the injection were not determined because the absence of uniform mixing makes the curves unreliable (14) Calculations made on the basis of the blood volume, the present dose and the concentrations show that nearly half the injected endotoxin is eliminated from the blood stream during this short pre-sampling period

Thus however, does not weaken the assumption that pretreatment with warfarin gives a higher clearance rate during the first 20 min after injection, the period in which the primary elimination of more than 75 per cent of the injected dose takes place The clearance rate is within the same range as that found by others in non tolerant rabbits (19, 38) The present experiment, however does not give any information about the possible dependency of the phagocytic rate on the injected dose (12) and on the duration of the warfarin pretreatment (1), or about the endotoxin induced depression of the R E S as determined by inert particle clearance (2, 25, 30)

The total amount of endotoxin in the examined organs from the warfarin treated group is somewhat higher than that from the untreated one Pretreatment with warfarin does not however, seem to give an altered pattern of endotoxin distribution No significant difference was found between any pair of organs, or between all organs, compared collectively Almost all the recorded amount of endotoxin is trapped or accumulated in the organs Only a small fraction of the amount represents endotoxin in circulating blood within the organs As the amount in the organs examined represents about 75 per cent and that in the peripheral blood about 25 per cent of the injected dose only a negligible fraction of the injected endotoxin is accumulated in other organs *e g* the bone marrow This agrees with the findings of *Braude et al* (5), but differs from those of *Herion et al* (19), who found about 15 per cent in the bone marrow

The present pattern of organ distribution of endotoxin does not diverge very much from what others have found with regard to the liver, kidneys, heart and spleen (19, 38) Noteworthy, however, is the very high percentage of the injected endotoxin in the lungs in the present experiment compared to previous reports (19, 38) According to *Braude et al* (5) the lungs had the highest endotoxin content per g tissue *Herion et al* (19) found that the spleen, liver and bone marrow had a higher content per g organ than the lungs The organ localization may be dosedependent, as recorded for injection of colloids (3, 12), although the time elapsed after injection is perhaps a determinant factor of more importance in this connection (3 38) Nevertheless, the present results may strongly indicate that the primary distribution is only very slightly influenced by the altered coagulability provoked by warfarin The experiment, however, gives no information about the influence of warfarin on the secondary release of endotoxin from the organs and the redistribution process (6)

The circulating endotoxin 20 min after injection is mostly located to the plasma phase, the small fraction bound to formed elements being the same in both groups This pattern was also obtained by *Braude et al* (5) and does not diverge much from the results of *Herion et al* (19) In untreated rabbits the dominant part of the fraction located to formed elements is bound to platelets and only a negligible part to leucocytes (2) Probably the same relationship is to be found in warfarin treated rabbits This suggestion may be supported by the equal pyrogenic response to endotoxin in untreated and warfarin treated rabbits (18)

The mechanism of the higher endotoxin blood removal rate provoked by warfarin is uncertain It may be based on competitive phagocytosis of fibrin clots and endotoxin It seems probable that warfarin treatment does

not influence the platelet binding of endotoxin, platelet interaction or the endotoxin-induced platelet aggregation (9) which may play the major role in the primary elimination of endotoxin from the blood (7, 20, 21 28)

Previously it has been found that the increased elimination rate provoked by warfarin has no influence on the pyrogenic response (18) or on the antibody formation (17) to endotoxin The changed elimination rate may be one factor in the inhibition of the Shwartzman reaction provoked by warfarin treatment It seems certain, however, that there are other factors of more importance, e g the influence of warfarin on the fibrin thrombi formation following the platelet aggregation (21, 27, 28), the fibrin localization (8), the redistribution of endotoxin (6) and the circulatory changes (8, 31) It is also possible that warfarin influences the still obscure detoxification process (35, 37)

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CONTENT IN CELL CULTURES OF COMPLEMENT ESTERASE AND TWO FURTHER ESTERASES MAINLY HYDROLYSING ETHYL ESTERS OF ACETYL-L-PHENYLALANINE AND ACETYL-L-TRYPTOPHAN, RESPECTIVELY

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Cell cultures of various origin—from cornea, cervix cancer, kidney, lung or skin from man monkey or rabbit—were found to synthetize complement esterase and two further esterases mainly hydrolysing ethyl esters of acetyl-L-phenylalanine and acetyl-L-tryptophan respectively Whether cells were tested directly after outgrowth in serum containing medium or after a prolonged period of maintenance in serum free medium or after years of growth in serum free medium they all contained high levels of all three esterases After intervals of time varying for different cell cultures either all three esterases or only one of them (C1s or acetyl phenylalanine esterase) also appeared in the serum free media nourishing the cells This release of esterases from cells into media was suppressed by cycloheximide Some evidence is also presented that C1s recovered from cells disrupted by sonication differed in esterolytic capacity from C1s found in the medium Finally an inhibition phenomenon was encountered, predominantly in cell extracts The phenomenon was temperature dependent affecting all three esterases at 37° C but almost not noticed at a test temperature of 25° C

The isolation from HeLa cell cultures of three esterases was recently described (23) One of these closely resembled human activated C1s showing similar esterolytic properties and also inactivating C4 Subsequent investigations have given additional support for the identity of this esterase and C1s Thus, the HeLa cell esterase in combination with C1q and C1r formed haemolytically active C1 (17) The esterase in question also behaved as human C1s in gel diffusion tests using specific anti serum against C1s (24)

In the present investigation some data based on esterolytic tests are presented indicating that C1s and the two other esterases found in HeLa cell cultures (here called acetyl phenylalanine esterase and acetyl tryptophan esterase) are formed also in a variety of cell cultures of human and animal origin

Received 21 x 71

MATERIALS AND METHODS

Cell cultures Primary cultures of Cynomolgus monkey kidney cells (pCMK), prepared according to the technique of *Bodian* (3) Serially propagated cultures (passage no 7–17) of diploid cells of human foetal lung prepared according to Hayflick and Moorhead (12) Continuously propagated cell cultures. Human origin HeLa cells of cervix carcinoma (9), NCTC 3075 cells of skin epithelium (1) Monkey origin GMK AH 1 kidney cells (10), Vero kidney cells (20), NCTC 3526 kidney cells (8) Rabbit origin Sirc corneal cells (13), RK 13 kidney cells (2)

Growth media The protein free NCTC 135 medium (8) was used to grow NCTC cultures 3075 and 3526

Eagle's Basal Medium (6) with Earle's balanced salt solution BSS, (7) supplemented with 10 per cent inactivated calf serum The medium was modified and contained about 3 times the original amount of L-arginine and twice that amount of L threonine and L-valine The following cells were grown in this medium HeLa diploid human foetal lung GMK-AH 1 Sirc and BHK-21

Medium 199 (16) with Hanks' BSS (11) supplemented with 10 per cent inactivated calf serum was used to grow Vero and RK-13 cells

Lactalbumine hydrolysate 0 5 per cent (15), in Hanks BSS supplemented with 2 per cent inactivated calf serum was the growth medium of pCMK cells.

The calf serum used as a supplement was filtered through Seitz EKS pads and then inactivated in 50 ml amounts in a water bath at 56° C for 45 min

Maintenance medium Medium 199 was used to maintain all grown out cultures except for NCTC 3075 and 3526 which were continously grown in NCTC 135 medium Autoclaved Bacto-tryptose (0 5 per cent) added to Medium 199 in previous experiments (21 22 23) was omitted since it made concentration and purification procedures more difficult

All media contained the following antibiotics per ml 100 IU penicillin 100 µg streptomycin and 50 µg neomycin

Culture flasks NCTC cultures 3075 and 3526 were grown in plastic flasks 75 cm² bottom area (Falcon Plastics California U S A) in 10 to 15 ml of medium in an atmosphere of 6 per cent CO_2 and 94 per cent air

For other cell cultures rubber stoppered Roux flasks of Pyrex glass (bottom area ca 220 cm²) were used throughout The amount of growth medium was 50 to 100 ml per flask Prior to addition of maintenance medium (40 ml per Roux flask) the out-growth medium was removed and cell sheets and inner surfaces of flasks were carefully washed 3 times with 100 ml of phosphate buffered saline (PBS)

Flasks were kept stationary at 35° C during incubation

Subcultures At passages cells of NCTC cultures were scraped off with a rubber policeman For other cultures the cell sheet of a flask was at subculture dispersed in 15 ml of one of the following agents (for details see ref 19)

A 0 02 per cent EDTA in PBS free from Ca^{++} and Mg^{++} This agent was used for HeLa cell cultures

B 0 25 per cent trypsin in PBS free from Ca^{++} and Mg^{++} (trypsin 1 300, Nutritional Biochemicals Corp, Cleveland, Ohio U S A) This agent was used for cultures of diploid human foetal lung cells

C 0 5 per cent trypsin and 0 04 per cent EDTA in PBS free from Ca^{++} and Mg^{++} This combination of agents was used for other cell cultures

Cells dispersed by EDTA were simply diluted with growth medium before the inoculation into new flasks Cells dispersed by trypsin (alone or in combination with EDTA) were spun down by low speed centrifugation and freed from supernatant before being resuspended in growth medium

Preparation of culture materials for enzymatic tests The medium of cultures was at harvest freed from cells by low speed centrifugation and stored at —35° C The further treatment included thawing in water of room temperature followed by centrifugation at 12,000 rpm for 60 min in a refrigerated centrifuge (MSE, High Speed 18, London England) The fluid was then concentrated 40 to 80 times on a Diaflo filter type XM 50 (Amicon Lexington, U S A) and equilibrated on the same filter against a 0 005 M phosphate buffer (pH 7 5) in 0 15 M NaCl by washing the concentrate twice with 10 volumes of the buffer Effluents from the XM 50 filter were treated in an analogous way on a Pellicon 1000 M W filter (Millipore Bedford Mass U S A) in order to test the XM 50 filter for leakage as well as to save small molecular material After clarification by low speed centrifugation the materials were distributed in small amounts into test tubes and kept in a Revco box at —75° C

The cell sheets of cultures were washed twice with PBS and after scraping with a rubber policeman suspended in a small volume (ca 25 ml per 10 flasks) of the above mentioned 0 005 M phosphate buffer After storage at —75° C the cell suspension was thawn and sonicated under ice cooling for 90 seconds at full effect in a MSE (London England) 100 W disintegrator The material was centrifugated at 30 000 rpm for 60 min in a Spinco type 30 rotor and the supernatant further treated as outlined above for the medium A cell extract was concentrated to about the same volume as was the medium which had nourished that particular amount of cells

All batches of media and cells were at the harvest

bacteriologically sterile also with regard to mycoplasmas

Cycloheximide was from Sigma Chemical Comp, St Louis, U S A

Synthetic amino acid esters These were obtained from commercial sources N-α-acetyl-L-lysine methyl ester HCl from Cyclo Chemical Corp, Los Angeles, U S A, N-acetyl L-phenylalanine ethyl ester from Sigma Chemical Comp, St Louis, U S A, N-acetyl-L-tyrosine ethyl ester from BDH Chemicals Ltd, Poole, England, N acetyl-L-tryptophan ethyl ester from Mann Research Lab, New York, U S A The esters of phenylalanine tryptophan and tyrosine were dissolved at a concentration of 0 5 M in methyl cellosolve (2 methoxyethanol Merck, Germany) Solutions were either made up daily or preserved in test tubes at —75° C The lysine ester was always prepared daily in 50 per cent methyl cellosolve phosphate buffer (0 005 M) The final concentration in assay was 0 02 M of ester and 4 per cent of methyl cellosolve

Esterolysis The equipment for measuring esterolysis was from Radiometer (Copenhagen, Denmark) One unit consisted of Titrator TTT 1c combined with Autoburette ABU 12 another unit was Titrator TTT 11, pH meter 26 and Autoburette 1c

Reactions between esters and esterases occured in the water jacketted 5 ml vessel of assembly TTA 31 The reaction volume was 2 5 ml and included varying amounts of the material to be tested for esterase, ester (0 1 ml) as well as additional buffer to make up for 2 5 ml (0 005 M phosphate buffer with 0 15 M NaCl) A constant pH of 7 5 was kept, acid liberated being neutralized by 0 05 N NaOH The amount of the latter as given by the burette was manually recorded at 1,3 or 6 min intervals for 60 min

Tests were carried out at 37° C and 25° C respectively

The existence in culture materials of some inhibitory factor(s) necessitates a specification of the order of adding esterase and ester, respectively to the reaction vessel In contrast to procedures followed earlier (23) the material containing esterase activity was in the present investigation with exceptions mentioned below added prior to the ester The reason for this was that large volumes (0 5 to 2 ml) of medium or cell extract containing low esterase activity needed prewarming for about 5 min to reach the test temperature

Esterase activity is expressed in micromoles of acid produced by the action of 100 ml culture medium (or extracts of cells grown in 100 ml medium) during a time period of 60 min The 60 min value given is, however calculated from an initial 6 min period as this represented the straightest part of the curve

Chromatography Some preparations were purified on columns of hydroxylapatite (Serva Germany) as described earlier (23)

Protein measurements were made with the Folin phenol reagent (14, 18) *)

RESULTS

Evaluation of esterases The properties of the three esterases found in HeLa cells are given in a previous paper (23) as ratios between the hydrolytic rates of various esters at 37° C These ratios were based on actual 60 min values of hydrolysis and not on 6 min values as used in the present investigation The ratios have therefore been recalculated for esters of current interest The new values (Table 1) differ only slightly from the ones previously presented Although the esters are not specific for the various esterases (the values for acetyl-tryptophan esterase moreover represented an insufficiently purified preparation) it is thought that the ratios given in Table 1 might serve as a key for a rough estimation of the content of the three esterases in various cell cultures the esterolytic properties of which are given i Tables 2, 3 4 5 and 6 An evaluation along these lines rests on the assumption that the properties for esterases from HeLa cell cultures (Table 1) are also valid for corresponding enzymes derived from other kinds of cell cultures since purified activities from the latter with a single exception have not yet been tested The justification of such an assumption has to some extent been corroborated by the separation, during the present investigation, of C1s from cultures of diploid human foetal lung cells, which fulfilled the criteria of this activity as to esterolysis (25) and haemolytic activity (17) as well as immunological identity (24)

Inhibitory factor(s) Curves on hydrolysis of the various substrates given by purified preparations of esterases deviated only slightly from linearity (Fig 1) There was no significant difference in this respect between results obtained at 37° C and at 25° C At the

*) These tests were kindly performed by Miss Britt Marie Lundin

latter temperature the reaction velocity was, as expected approximately half as high as at 37° C

The rate of hydrolysis brought about by cell extracts, on the other hand, rapidly decreased at 37° C (Fig 2) The initial reaction velocity at this temperature was in a few minutes reduced to about the same level as that recorded at 25° C Although the rate of hydrolysis at 37° C continued to decrease the reaction was seldom brought to zero during the test period In contrast, cell extracts at 25° C hydrolysed at a rate which only moderately decreased with time

The inhibition of hydrolysis at 37° C was pronounced in all cell extracts tested (Tables 2, 3, 4, 5, and 6) and often lower values for hydrolysis were found at 37° C than at 25° (especially in the case of the acetyl-tyrosine ester) This may partially be due to the pre warming of materials (see Methods) as this when brought to 37° C obviously permitted

TABLE 1 *Esterolytic Ratios of Three Esterases from HeLa Cell Cultures*

Esterases	Esters			
	N acetyl L-tyrosine Ee	N-α acetyl L-lysine Me HCl	N acetyl L phenylalanine Ee	N acetyl L-tryptophan Ee
H 2 DS* = Cls †	1 §	2 95	0 13	0 21
H 1* = acetyl phenylalanine esterase †	0 1		1§	–
H 3* = acetyl tryptophan esterase †	0 42	0 33	0 33	1§

* Previously (23) used designation
† Presently used designation,
§ Marks reference ester of the various enzymes

Ee = ethyl ester
Me = methyl ester
– = no hydrolysis

TABLE 2 *Esterases in Cultures of HeLa Cells*

Material and day of harvest (after the change of cultures to serum free medium)		Protein, mg per 100 ml medium* or cell extract*	Micromoles of H⁺ liberated per hour by 100 ml medium or extracts of cells grown in 100 ml medium							
			N-acetyl L tyrosine Ee		N-α-acetyl L lysine Me HCl		N acetyl L phenylalanine Ee		N acetyl L-tryptophan Ee	
			25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Control series										
Medium	1 d	0 4	<1 4	<2 8	<1 4	<2 8	<1 4	<2 8	<1 4	2 8
Medium	4 d	7 9	8 4	25 2	21	39 9	12 6	31 5	5 6	10 5
Cell extr	1 d	13 4	45	45	5 3	7 9	66 3	88	21 2	34 5
Cell extr	4 d	7 8	29 2	25	5 6	8 4	37 5	45 8	13 9	16 7
Series with 5γ cycloheximide per ml medium										
Medium	4 d	1 6	<1 3	2 5	<1 3	2 5	<1 3	2 5	<1 3	2 5
Cell extr	4 d	10 2	21 1	21	1 3	7	28 2	56	11 9	19 3

Flasks of these series were incubated for indicated time periods without further change of medium
* As calculated from measurements on materials used in esterolytic tests. Abbreviations, see Table 1

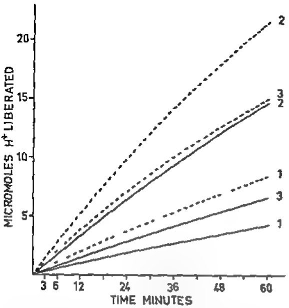

Fig 1 Hydrolysis by esterases purified from media of HeLa cell cultures on a column of hydroxyl apatite
1 = hydrolysis of N–acetyl–L–tyrosine ethyl ester by C1s,
2 = hydrolysis of N–α acetyl–L–lysine methyl ester by C1s,
3 = hydrolysis of N–acetyl–L–phenylalanine ethyl ester by acetyl phenylalanine esterase
The recording of hydrolysis started immediately after the addition of esterase Dotted lines 37° C Solid lines 25° C

inhibitory factor(s) to react with esterases prior to the recording of esterolysis The inhibitory phenomenon was not influenced by the time of harvest of cells Thus extracts of freshly grown out cells behaved as extracts of cells maintained for four weeks on serum-free medium, the latter being changed weekly

The activities of media from cell cultures were in general only moderately influenced by inhibitory factor(s) and the hydrolysis proceeded at a higher rate at 37° C than at 25° C (Tables 2, 3, 4, 5 and 6) The rates sometimes were twice as high at the former temperature but more often of an intermediate order, the latter suggesting a contamination of media to a varying degree with inhibitory factor(s) released from spontaneously lysed cells

Due to the temperature dependent inhibition described above comparisons between the various materials as to the content of esterases obviously must be based on tests performed at 25° C This was, however, not always possible (Tables 3 and 4)

Content of esterases in cell extracts The esterolytic ratios given for C1s in Table 1 between the ester of tyrosine relative to the esters of phenylalanine and tryptophan were compared with the ratios between the values of the various cell extracts presented in Tables 2, 3 4, 5 and 6 All cell extracts, irrespective of time of harvest, showed a 7 to 11 fold higher rate of hydrolysis of the phenylalanine ester and a 2 to 3-fold higher hydrolysis of the tryptophan ester than given by C1s Cell extracts of Vero, pCMK, RK–13, NCTC 3075 and NCTC 3526 behaved similarly

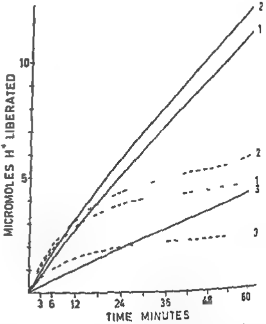

Fig 2 Hydrolysis by an extract of GMK cells harvested after an incubation period of 25 days on serum free medium A similar material is represented in test B of Table 5
1 = hydrolysis of N acetyl L–tyrosine ethyl ester
2 = hydrolysis of N–acetyl L phenylalanine ethyl ester,
3 = hydrolysis of N–acetyl L tryptophan ethyl ester
The recording of hydrolysis started immediately after the addition of cell extract Dotted lines 37° C Solid lines 25° C

The high hydrolysis of the phenylalanine and tryptophan esters indicates the presence in all cell extracts tested of the enzymes referred to in Table 1 as acetyl phenylalanine esterase and acetyl tryptophan esterase

Contrary to expectation the hydrolysis of the tyrosine ester, which should be mainly attributable to C1s, was in cell extracts not connected with the relatively higher hydrolysis of the lysine ester shown for C1s in Table 1 Instead, ratios between the hydrolysis of tyrosine and lysine esters ranged from 1 0 05 to 1 0 19 Some cell extracts (not shown in the tables) did not hydrolyse the lysine ester at all yet such a HeLa cell extract contained significant amounts of C1s which gave haemolytically active C1 when combined with C1q and C1r (17) Thus it seems probable that the activity of cell extracts against tyrosine ester represents a form of C1s with different esterolytic capacity

Although ratios between hydrolysis of the various esters were closely similar for the various cell extracts, cell cultures differed quantitatively in hydrolytic capacity as is illustrated by the amounts of micromoles H^+ liberated per hour at 25° C from N-acetyl-L-tyrosine ester by extracts corresponding to 10 mg of initial cell protein The following order was obtained (the amount of acid given within parenthesis) diploid human foetal lung (58),

TABLE 3 *Esterases in Cultures of Diploid Human Foetal Lung Cells*

Material and day of harvest (after the change of cultures to serum free medium)		Micromoles of H^+ liberated per hour by 100 ml medium or extracts of cells grown in 100 ml medium							
		N acetyl L-tyrosine Ee		N-α acetyl L-lysine Me HCl		N acetyl L-phenylalanine Ee		N-acetyl L-tryptophan Ee	
		25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Control series									
Medium	1 d		26.3		61.3		4.4		6.6
Medium	2 d		34.7		84.4		5.7		11.3
Medium	3 d	23.4	45.2	61.5	96.2		7.3		7.3
Medium	4 d	40	63.4	99.8	148.4	6.7	10		16.7
Medium	6 d	64.6	117.5	212	340.8	13.7	23.5		29.4
Cell extr	6 d	43.8	41.7		6.3		42.8		31.3
Series with 10γ cycloheximide per ml medium									
Medium	3 d		0.9		9.5		2.9		0.9
Followed by 2 further days without cycloheximide									
Medium	= 5 d		17.2		41.2		12.6		10.3
Followed by 2 further days without cycloheximide									
Medium	= 7 d	17.8	29.5	39.1	8? 6		10.6		10.6
Cycloheximide reinstated for 4 days									
Medium	= 11 d		4.8		9.6		7.7		2.9
Cell extr	11 d	26.3	21.3		3.2		21.3		11.7

Flasks of the control series were incubated for indicated time periods without further change of medium. Abbreviations see Table 1

GMK (32), NCTC 3526 (26), HeLa (14) and NCTC 3075 (8).

Content of esterases in media. In cultures of HeLa cells kept for a week without change of medium esterase activities appeared in the media after one to two days of incubation. Media collected a few days later contained *from one third (Table 2) up to the same* amount of esterases as did the cells. The latter were even surpassed as a source of esterases by media harvests of the 6th day. At this time, however, many cells were detached from the glass and further culturing had to be discontinued.

As in HeLa cultures esterolytic activities of media from diploid human foetal lung cells increased from the first to the 6th day harvest (Table 3). The cultures were maintained in a good condition with weekly changes of media, the esterase contents of which were high during a long period of time (Table 4).

Media of GMK cultures incubated for one week contained either no or only traces of esterolytic activities (Table 5). Increasing amounts of such activities were found in media harvests of the second to the fourth week. However, at the latter time the esterase content of cells still was higher than the total amount of activities recovered from media during the whole test period.

Only small amounts of esterolytic activities were found in media of Sirc cultures (Table 6) during the first two weeks of incubation. In later harvests hydrolysis of the phenylalanine ester markedly increased.

Media hydrolysed *tyrosine ester relative* to the esters of lysine, phenylalanine and tryptophan (expressed as ratios between the esters in the order mentioned) as follows:

Media of HeLa cell cultures, 1 2.5 1.5 0.67, media of diploid human foetal lung cell cultures, 1 *2.3–2.7 0.14–0.31 0.19–0.39* media of GMK cell cultures 1 2.2 2.4 0.20–0.27 0.05–0.23.

TABLE 4. Esterases in Cultures of Diploid Human Foetal Lung Cells

Test	Material and day of harvest (after the change of cultures to serum free medium)		Protein mg per 100 ml medium* or cell extract*	Micromoles of H liberated per hour by 100 ml medium or extracts of cells grown in 100 ml medium			
				N acetyl L tyrosine Ee 37°C	N α acetyl L lysine Me HCl 37°C	N acetyl L phenylalanine Ee 37°C	N acetyl L tryptophan Ee 37°C
A	Medium	6 d	1.7	48.4	110.2	14.8	10.9
A	Medium	12 d	1.0	64.2	166	9.1	12.1
A	Medium	18 d	0.6	41.4	113.1	10.2	9.5
A	Medium	25 d	0.5	39.5	107.7	6.5	10.1
A	Medium	32 d	0.3	16.9	50.7	4	3.6
A	Medium	39 d	0.2	13.5	37.3	1.9	1.9
A	Medium	46 d	0.2	18	43.1	4.7	7.1
B	Medium	6 d		80	241.7	10	16.7
B	Medium	12 d		49	142.5	11	11.5
B	Medium	18 d		48	145	9	9
B	Medium	24 d		50.4	148.7	10.7	12.2
B	Medium	31 d		52	141.8	11	7.9
B	Medium and cell extr	38 d		54.3	122.3	38.5	21

Flasks of tests A and B were incubated until the final harvest. The medium was changed about once a week.

* As calculated from measurements on materials used in esterolytic tests. Abbreviations see Table 1.

In media of cell cultures just referred to ratios between hydrolysis of tyrosine and lysine esters were in agreement with the ratio given by Cls (Table 1) This enzyme apparently was responsible for most of the esterolytic activities in media from cultures of diploid human foetal lung cells and GMK cells Media of HeLa cell cultures besides Cls, should contain acetyl phenylalanine esterase and acetyltryptophan esterase as the amino acid esters

TABLE 5 *Esterases in Cultures of GMK Cells*

Test	Material and day of harvest (after the change of cultures to serum free medium)		Protein, mg per 100 ml medium* or cell extract*	Micromoles of H⁺ liberated per hour by 100 ml medium or extracts of cells grown in 100 ml medium							
				N acetyl L-tyrosine Ee		N α-acetyl L-lysine Me HCl		N acetyl L phenylalanine Ee		N acetyl L tryptophan Ee	
				25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
A	Medium	1 d		<2	4	<2	6	<2	10	<2	6
A	Medium	3 d		<2	<1 8	<2	<1 8	<2	4 4	<2	<1 8
A	Medium	6 d		<2 3	<2 3	<2 3	<2 3	<2 3	<2 3	<2 3	<2 3
A	Cell extr	1 d		82 8	67 9	<4 6	13 8	96 6	87 4	48 3	47 2
A	Cell extr	3 d		104	94	6	7	108	104	56	50
A	Cell extr	6 d		72	73 2	<4 5	9	90	74 3	47 3	38 3
B	Medium	6 d	1 4	<0 7	1 5	<0 7	3 6	2 9	6 5	<0 7	1 5
B	Medium	12 d	2 3	11 9	22 8	26 1	44 5	2 7	7 6	0 6	6 5
B	Medium	18 d	2 8	31 5	40 5	74 3	94 5	8 6	10 5	7 2	9
B	Medium	25 d	3 3	23 4	32 8	52 7	77 2	4 7	6 5	4 7	7
B	Cell extr	25 d.	4 8	90 9	71 7	4 2	10 1	104 1	81 5	43	36 1

Flasks of test A were incubated for indicated time periods without further change of medium
Flasks of test B were incubated until the final harvest The medium was changed about once a week
* *As calculated from measurements on materials used in esterolytic tests*
Abbreviations see Table 1

TABLE 6 *Esterases in Cultures of Sire Cells*

Test	Material and day of harvest (after the change of cultures to serum-free medium)		Micromoles of H⁺ liberated per hour by 100 ml medium or extracts of cells grown in 100 ml medium							
			N acetyl L tyrosine Ee		N α acetyl L lysine Me HCl		N-acetyl L phenylalanine Ee		N acetyl L tryptophan Ee	
			25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
A	Medium	6 d	<0 8	3 8	<0 8	2 7	3 2	14	<0 8	<0 6
A	Cell extr	6 d	32 2	30 5	4 6	6 4	42 6	60 9	13 3	17 9
B	Medium	6 d	3 9	7 9	<0 9	8 8	4 9	17 7	1 9	5 9
B	Medium	12 d	3 9	4 7	<0 8	3 9	4 7	14	3 1	4 7
B	Medium	19 d	3	6	<0 8	3	13 5	33 8	2 3	1 5
B	Cell extr	19 d	33 9	38 6	<0 9	4 6	48 7	77	17 5	16 5

Flasks of test A were incubated for indicated time periods without further change of medium.
Flasks of test B were incubated until the final harvest The medium was changed about once a week
Abbreviations see Tabel 1

indicating these enzymes were strongly hydrolysed. The correctness of these conclusions in the case of HeLa cell medium was proved by tests on purified enzyme preparations (23). During the present investigation C1s̄ was purified from media of diploid human foetal lung cells (25). From columns used in this connection none of the other activities was recovered indicating that only small amounts of these may have been present in the starting material.

Media of other cell cultures tested. Like in GMK, media from cultures of Vero and pCMK predominantly contained C1s. In Vero high amounts of this activity were found already in one week harvests. Similar to cultures of Sirc cells media from Rh 13 cultures were rich in acetyl phenylalanine esterase appearing already in early harvests. Media of NCTC cultures 3075 and 3526, in addition to C1s contained relatively high amounts of acetyl phenylalanine esterase (ratios 22 and 12 times higher, respectively, than given by C1s in Table 1) and acetyl tryptophan esterase (ratios 10 and 2.5 times higher, respectively, than given by C1s̄ in Table 1).

Cycloheximide tests. The incorporation of cycloheximide (5γ/ml) into the medium of HeLa cells (Table 2) obviously suppressed the release of all three activities from the cells. Cycloheximide (10γ/ml) had a similar action on diploid human foetal lung cells (Table 3). A removal of the substance resulted in a marked increase of C1s in media.

DISCUSSION

The capability of HeLa cells grown in serum free medium to synthetize C1s and an enzyme here called acetyl phenylalanine esterase was reported on earlier (23). A synthesis of these enzymes and an additional one here called acetyl tryptophan esterase was in the present study demonstrated to occur in two cell cultures established years ago to grow in a protein free medium (NCTC 135). None of the above cell cultures were dispersed with trypsin during the cultivation procedures.

The evidence for a synthesis of esterases by the other cell cultures of this investigation needs to be discussed as the technique of growing these cultures included trypsin as a dispersing agent and serum as an essential nutritive. These substances are undesirable in systems intended for a study of the biosynthetical capacities of cells. Trypsin activity however, most probably was removed by washing, addition of serum and thermal inactivation upon incubation of cultures. Serum furthermore, which might serve as a source of enzymes and inhibitors to be adsorbed by the cells probably was rendered harmless in this respect by heat treatment. In addition cells were tested for esterases only after a careful washing. Furthermore the cultures were kept for long periods of time on serum free medium changed at intervals. After several weeks of such a prolonged washing the cells still contained amounts of esterases comparable to those of freshly harvested cells. Finally the release of esterases from cells into the media during the maintenance of cultures was suppressed by cycloheximide, a fact which strongly argues against a hypothesis of a desorbtion of esterases from the cell surfaces. It is therefore concluded that the three esterases referred to above were synthetized by the cells also in cultures which required serum for outgrowth.

The results of the investigation indicated as could be expected, that the main synthesis of esterases in serum dependent cultures probably occured during the growth phase (with serum) at the end of which cells seemed to have a maximum content of enzymes.

There were however indications of an enzyme synthesis in some cell cultures also during the maintenance period when cells were kept on serum free media at a low metabolic level. Thus in diploid human foetal lung cells serial harvests of media give a total recovery of C1s̄ which was three to four times higher than the maximum amount of the enzyme found at one time in cell extracts i. e. in freshly grown out cells of this kind. In other cultures cells showed a high content of enzymes over long periods of time. A synthesis

of enzymes may be involved in such cases too although masked by a correspondingly high decay

The keeping of cultures on serum free media for prolonged periods of time also made possible a comparison between the Cls enzyme spontaneously released from cells into the medium and that extracted from cells by sonication In the latter case the enzyme appeared to have no or a very low activity on the acetyl lysine ester and would hence not have been recognized as Cls on the basis of the esterolytic pattern only Further investigations are however needed to clarify this matter

In many cultures the set of enzymes differed in media and cells Cls was the only enzyme found in culture media of GMK Vero pGMK and diploid human foetal lung cells Acetyl phenylalanine esterase dominated esterolysis in media of Sirc and RK–13 cultures These findings indicate that enzymes were selectively released into the media from cells as the latter in all cultures contained three esterases

Finally, comparisons between curves of hydrolysis given by media and cell extracts led to the detection of some inhibitory factor(s) predominantly present in cell extracts and affecting the hydrolysis by all three esterases at 37° C but almost not at 25° C

The enzymes acetyl phenylalanine esterase and acetyl tryptophan esterase are apparently not described in the literature except for in a previous work by the author (23)

As regards Cls little is known about the formation of this compound in body tissues *Colten et al* (4) have only found a synthesis of haemolytic Cl (which must include Cls) in tissues from the small intestine of guinea pigs as well as in tissues of human colon and ileum (5) In the case of Cls a synthesis limited to intestinal cells would not be in agreement with the results of the present investigation On the contrary Cls as well as the two other esterases probably are widely spread cellular enzymes of animal tissues Thus they were found in cell cultures of different origin (cornea cervix cancer kidney lung and skin) from various mammals (man, monkey and rabbit) The stable existence in the cells was illustrated by the demonstration of the enzymes in question in freshly established cell cultures as well as in cultures subjected to hundreds of passages for periods of many years

The skilful technical assistance of Miss *Siri Austrin*, Miss *Tanja Sundberg* (tissue cultures) and Mr *Nils Broling* (biochemical procedures) is gratefully acknowledged

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LABELLING OF DNA AND RNA FROM THYMINE AND THYMIDINE IN *NEISSERIA MENINGITIDIS*

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Radioactivity was taken up from labelled thymine and thymidine by whole cells of *Neisseria meningitidis* strain M1, and both DNA and RNA were labelled The labelling from sub strates carrying the radioactivity in the C 2 position and in the methyl group was compared The uptake of radioactivity from thymine uniformly labelled, and from thymidine with label in the 6 position was also measured The incorporation of radioactivity varies with the location of the label in the molecule, and the uptake kinetics for the compounds with the label in the C 2 position is different from the uptake kinetics of the compounds with label in other positions The findings point to a utilization of break down products from thymine and thymidine

Attempts to isolate thymine, thymidine and TMP deficient mutants in *Neisseria meningitidis* for specific labelling of DNA were altogether negative This led to an examination of the uptake of these compounds when exogenously added The experiments showed that thymine uniformly labelled with ^{3}H was taken up to an extent of 5 per cent thymidine labelled with ^{3}H in the 6 position to an extent of 0 6 per cent, whereas the uptake from TMP was insignificant (15, 16) These findings agree well with those reported for thymine and thymidine by *Kingsbury & Duncan* (18)

Subsequently, several enzyme functions known to be involved in the specific incorporation of thymine and thymidine into DNA were searched for (1, 15) Activities corresponding to thymidine phosphorylase EC 2 4 2 4 or nucleoside deoxyribosyl transferase EC 2 4 2 6 could not be found, and no significant thymidine kinase activity EC 2 7 1 21 could be induced in the test strains

But, *N meningitidis* extracts had activities corresponding to TMP and TDP kinases EC 2 7 4 4 and EC 2 7 4 6 although whole cells which were exposed to TMP methyl ^{3}H did not take up significant amounts of this compound

Exogenously added radioactive thymine or thymidine does indeed result in some labelling of *N meningitidis* cells, although the experimental evidence indicates that the substances are not incorporated directly into TMP, TDP or TTP (1, 15, 16) Therefore, it appears likely that the substances are broken down before their radioactivity is utilized As a consequence uptake of thymine or thymidine should probably not result in a specific labelling of DNA in this microbe The present work was performed to elucidate these problems

Utilization of exogenously supplied thymine or thymidine after breakdown would be ex

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pected to give different results according to the place of the label in the molecule (6,7,8). Accordingly, the uptake of radioactivity from thymine and thymidine labelled in the methyl group was first compared with uptake from the same compounds carrying the label in the C-2 position.

To find an answer to the practical question whether radioactive thymine or thymidine may be used to obtain specific labelling of DNA under these circumstances, in spite of the lack of enzymes that are known to mediate such incorporation in other microbes or mammalian cells, the labelling obtained in DNA and RNA was subsequently measured.

MATERIALS AND METHODS

The media and methodology employed in this investigation were analogous to those previously used (15, 16). The bacterial strains were also the same, with *N. meningitidis* strain M1 of group B as the main test microbe. The maximum growth rate of this strain in the KC medium was 1.4 generations per hour under the conditions employed.

Incorporation into acid precipitable material. Incorporation of labelled material into acid precipitable cellular constituents was measured as previously described (16). An overnight culture on blood agar was harvested in the KC medium. The suspension was grown to the logarithmic growth phase before aliquots with about 8.5 × 10⁸ colony forming units per ml were mixed with the radioactive material to be tested. The filters were counted in a toluene based scintillation fluid in the *Packard Tri Carb Spectrometer (15) with a counting* efficiency of the ^{14}C material of 79 per cent and of the ^{3}H material of 22 per cent.

Labelling of nucleic acids. Fractionation of the labelled cells was first performed by a modification of the Schmidt Thannhauser procedure as recommended by *Munro & Fleck* (22). 12 ml KC medium was [illegible]
to the log [illegible]
cells/ml), [illegible]
^{14}C 2 thy[illegible]
and shake [illegible]
was cooled in an ice water bath, the cells were collected by centrifugation and resuspended and washed four times with ice cold KC medium. The cells were subsequently suspended in 6 ml distilled water at 0° C and frozen and thawed twice to help disrupt the cells. The lipid extraction step was omitted (11, 22). *Four parallels of 1.25 ml of the cell suspension were then extracted at* [illegible]° C for 10 min with 0.2 N $HClO_4$ *final concentration.* The residue was washed twice with 0.2 N $HClO_4$ and next the RNA was extracted with 0.5 N KOH at 37° C for 5 h. After a series of experiments this *procedure was found to* give a complete and uniform hydrolysis in all samples. The DNA and protein were precipitated at 0° C with $HClO_4$ (0.2 N final conc.), and after washing the DNA was extracted with 0.5 N $HClO_4$ at 70° C for 2 h. (13) RNA was determined by the orcinol reaction with purified orcinol recrystallized from benzene and with ribose as a standard (4, 21). DNA was estimated by the procedure of *Burton* (2) with *2-deoxy-D-ribose* as a standard. Samples of each fraction were counted in the Packard Tri-Carb Spectrometer with 10 ml Insta gel (Packard Instrument Co. Inc.) as *the scintillation fluid* and they were *corrected for background.* The counting *efficiency was found to be 76 per cent.* No quenching correction was found to be necessary.

Examination of the *distribution* of label among various cell constituents was also performed by a method based on nuclease treatment of the extracts (10, 24, 25). Cultures in 3 ml of minimal medium (Medium A) (16) in the logarithmic growth phase (*appr. 4 × 10⁸ cells/ml) were grown* with shaking at 37° C for 105 min with a total of 6.25 µg [illegible] thymine uniformly labelled or 9.7 µg ^{3}H thymidine-6-T(n). The cultures were centrifuged at 4° C and washed three times with ice cold *SSC buffer* (NaCl, 0.14 M, Na_3 citrate 0.015 M) *pH 7.4*. The cells were suspended in *3 ml of the SSC* buffer and treated in a M.S.E. ultrasonic disintegrator (11, 15). The cell debris *was removed by* centrifugation at 7900 × g for 15 min. *Aliquots of* the supernatant, usually *100 and 25 µl* were taken for acid soluble, acid *precipitable deoxyribonuclease* and ribonuclease hydrolyzable material. *Samples for* initial acid soluble and acid precip*itable* material were mixed with an equal volume *of ice cold 10 per* cent trichloroacetic acid (*TCA*) *with an additional* ice cold 3 ml 5 per cent *TCA* and *kept in* ice water for 30 minutes. The rest of the *procedure* was as *described previously except that the washing* of the filters with water was omitted (16). Radioactivity of the acid soluble *material* was determined by counting dried *samples in the* planchets. No self absorption correction was *found necessary.* Separate aliquots were treated with *deoxyribonuclease* (12) and ribonuclease for *30 min* at 37° C. The concentration was *50 µg DNase* or RNase per *ml* supernatant (*RNase 2 mg/ml* in SSC *buffer* was heated to *80–90° C for 10 min* before use to destroy deoxyribonucleases). The precipitation, filtration and *counting of* the material was as described. DNA and RNA were estimated as *the* difference between the acid *insoluble* fraction and the DNase respectively the RNase non hydrolyzable fraction. Determinations were *corrected* for endo-

genous breakdown to acid soluble material and for background

The labelling of DNA and RNA was also compared after isolation and separation by density gradient centrifugation. Radioactive material was added to the test microbe logarithmically growing in the KC medium (1.7×10^9 colony forming units per ml), and the culture was incubated with shaking at 37° C for 30 min. ^{14}C–2 thymine or ^{14}C–2 thymidine was added to 10 ml cultures at a concentration of 4.1 μg per ml and 7.8 μg per ml respectively. Thymine–methyl–^{3}H or thymidine–methyl–^{3}H was added to 14 ml culture at concentrations of 5.3 and 4 μg per ml. The incubation was terminated by cooling in an ice-water bath, and the cells were collected by centrifugation at 4° C. The cells were washed five times with ice cold KC medium, 3 ml at the time, and suspended in 2.4 ml SSC buffer pH 7.4. Sodiumdodecylsulphate 0.06 ml 5 per cent was added to the suspension which was then incubated for 15 min at 56° C and next left at room temperature for 30 min. In most experiments 0.6 ml of this crude extract was run in the CsCl gradient. The rest of the crude extract was treated according to *Marmur* (20) with the modification used in transformation experiments (5). The precipitated nucleic acids were resolved in the SSC buffer pH 7.4 and 0.6 ml was run in the CsCl gradient as described below.

Density gradient centrifugation. Samples of 0.6 ml nucleic acid solution were mixed with 2.4 ml stock CsCl solution in polyallomer tubes according to *Lacks et al.* (19). The ethanol precipitated nucleic acid solutions contained in 0.6 ml 90 to 170 μg nucleic acids. This was measured by spectrophotometry after suitable dilution. An absorbance at 260 mμ of 1.0 was taken to be equivalent to 50 μg DNA plus RNA per ml (9, 19). Stock solutions of CsCl were prepared by dissolving 1.1729 g CsCl to a total of 1 ml with SSC buffer pH 7.4. The amount of CsCl was estimated in accordance with the density of the buffer and the nucleic acid solution. This will result in a final density very close to 1.710 g/cm³ (13). Tube contents were overlayered with 2 ml paraffin oil and the samples were centrifuged in a Spinco model L centrifuge at 37,000 R.P.M. for 70 hours at 5° C using an SW 39 L head. Tubes were pierced and 3–6 drops fractions (0.054 to 0.096 ml) were collected. Each fraction was diluted with 1 ml of distilled water and the absorbancy read at 260 mμ in a Hilger Gilford spectrophotometer. All samples were counted with liquid scintillation spectrometry. The scintillation fluid was 10 ml Insta gel.

Chemicals. ^{14}C–2 thymine 60 mCi/mmole, ^{3}H thymine T(G) 1000mCi/mmole, thymine methyl ^{3}H 1000 mCi/mmole, ^{14}C–2 thymidine 58 mCi/mmole and 57 mCi/mmole, ^{3}H thymidine–6–T(n) 5000 mCi/mmole, thymidine–methyl–^{3}H 5000 mCi/mmole, were all obtained from the Radiochemical Centre, Amersham, Bucks, U.K. D ribose, 2–deoxy–D ribose, batch 13275, deoxyribonuclease 1 (DNase bovine pancreas), CsCl 99.9 per cent, were obtained from Koch Light Labs., Ltd. Bucks U.K. Ribonuclease (RNase bovine pancreas crystallized) was from Sigma Chemical Corporation, St. Louis, Mo. U.S.A. Orcinol was obtained from BDH Chemicals Ltd. Poole, U.K.

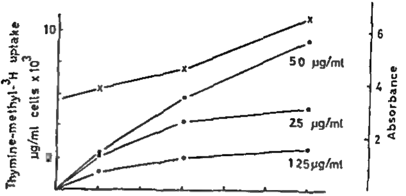
RESULTS

Incorporation of Radioactivity from Thymine and Thymidine Labelled in Different Parts of the molecule. Incorporation was first registered in the acid precipitable material from differently labelled thymine and thymidine. Fig. 1 and Fig. 2 show the rates of uptake of these compounds as well as the dependence upon the concentration.

Whereas ^{3}H thymine uniformly labelled is incorporated to an extent of 5.6 per cent (16), the incorporation from thymine methyl tritium is only to an extent of 0.2 per cent under the conditions employed. But as for the uniformly labelled substrate (16), the uptake of radioactivity from thymine methyl tritium is approximately proportional to the concentration of the substrate, and increases significantly with the time of incubation (Fig. 1).

Also the uptake of radioactivity from ^{14}C–2–thymine is far less than from the uniformly labelled substance being maximum 0.3 per cent. But in the case of the ^{14}C–2–thymine the uptake kinetics is apparently entirely different, since the uptake is not a function of the concentration in the same way, and the incorporation is little influenced by the time (Fig. 1).

When ^{3}H–thymidine–6–T(n) was the substrate the per cent incorporation was maximum 0.6, and the incorporation was a function of the concentration (16). In contrast, the incorporation from thymidine methyl tritium is reduced to a maximum of 0.02 per cent under similar conditions. In both instances, however, the incorporation is a func-



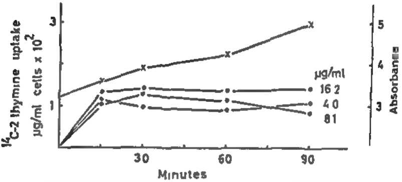


Fig 1 Utilization of exogenous ^{3}H-methyl and ^{14}C-2-thymine by *N meningitidis* Influence of the time of incubation The culture was grown logarithmically in the KC medium and portions of the culture were mixed with the labelled material and shaken at 37° C Samples were removed and assayed for acid precipitable radioactivity (×——× = Growth, ●——● = Incorporation)

tion of the concentration, and of the time of incubation (Fig 2)

The labelling of *N meningitidis* from ^{14}C-2-thymidine reaches a maximum of around 0.05 per cent which is of the same dimension as that obtained from thymidine methyl tritium But as for ^{14}C-2-thymine, the labelling is little influenced by the time of incubation Also, the incorporation is only moderately influenced by the concentration (Fig 2)

Labelling of nucleic acids from radioactive thymine and thymidine Several experiments were next performed to find out to what

TABLE 1 *Distribution of ^{14}C Among Fractions Derived from Cells of* N meningitidis *Strain M1 Grown in Medium KC Containing ^{14}C 2-thymine or ^{14}C 2 thymidine*

Compound	Quantity added, µg	Per cent of label incorporated total	Per cent of label added initially			Ratio of label in RNA/ label in DNA	Ratio of RNA/DNA	Growth rate gen./hour
			Acid sol	RNA	DNA			
C-2-thymine*	48,15	0.013	0.00023	0.0100	0.0016	6.4	9.71	1.1
C-2-thymidine*	94,50	0.014	0.00042	0.0087	0.0021	4.1	9.99	1.1

The cells were fractionated by a modification of the Schmidt Thannhauser procedure as described in Methods * Figures average of four parallels

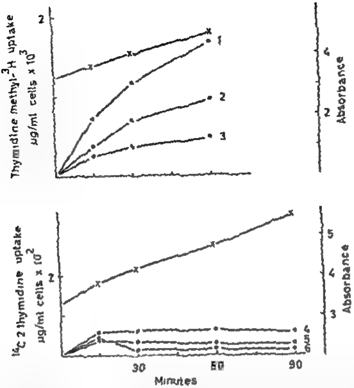


Fig 4 Utilization of exogenous ^{3}H methyl and ^{14}C–2–thymidine by *N meningitidis* Influence of the time of incubation Curves 1 2 and 3 with 7 7, 4 4, and 2.2 μg thymidine-methyl-^{3}H per ml cell culture respectively Curves 4, 5, and 6 with 32 3, 16 2 and 8 1 μg ^{14}C–2–thymidine per ml cell culture The rest of the conditions of the experiments as in Fig 1 (×——× = Growth ●——● = Incorporation)

extent the incorporation of radioactivity from labelled thymine or thymidine would result in a specific labelling of DNA

When *N meningitidis* strain M1 was grown in the presense of ^{14}C–2–thymine or ^{14}C–2–thymidine the isotope was recovered in fractions corresponding to RNA as well as to DNA (Table 1) The ratio of label in RNA

TABLE 2 *Relation between RNA and DNA Content in* N meningitidis *Strain M1*

Exp no	Medium used	Growth rate gen /hour	Sample no	RNA amt μg/sample	DNA amt μg/sample	Ratio RNA/DNA avg
1	KC	1 0	1	169 55	16 76	10 05
			2	175 60	17 49	
			3	175 06	17 49	
2	BH	1 9	1	221 40	15 85	15 02
			2	223 46	14 03	
			3	229 76	14 94	
			4	225 90	15 12	

The cells were grown logarithmically in the Medium KC and in brain heart infusion broth (BH, Difco) The cells were fractionated by a modification of the Schmidt Thannhauser procedure

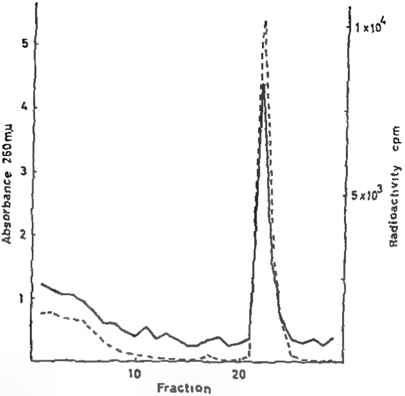


Fig 3 CsCl banding of material in *N meningitidis* containing radioactivity from thymine-methyl-^3H Cells exposed to thymine for 30 min Lysed extract deproteinized and ethanol precipitated Nucleic acids dissolved in SSC buffer (—— = Absorbance, - - - Radioactivity)

to that recovered in DNA has been compared with the actual RNA DNA ratios under the same cultural conditions For comparison *the ratio of RNA DNA in a culture* grown in brain heart infusion broth (BH, Difco) has been included (Table 2)

To find out to what extent the incorporation into DNA and RNA accounts for the total incorporation into the acid insoluble material, analysis was also performed by a method based on solubilization after nuclease treatment (24, 25) Table 3 shows that only 30 per cent of the label in the initial acid insoluble fraction became acid soluble after treatment with deoxyribonuclease when cells were grown in the basal medium (*Medium*

TABLE 3 *Distribution of ^3H among Fractions Derived from Ultrasonic Extracts of* N meningitidis *Cells Grown in Medium A Containing ^3H thymine T(G) or ^3H-thymidine-6 T(n)*

Compound	Quantity added μg	Per cent of label incorporated total	Per cent of label added initially			Ratio of label in RNA/label in DNA	Growth rate gen /hour
			Acid sol	RNA	DNA		
^3H thymine–T(G)*	6 25	5 64	1 580	2 800	1 230	2 28	0 7
^3H-thymidine–6 T–(n)*	9 70	0 52	0 318	0 104	0 077	1 35	0 7

The crude extracts were taken for acid soluble acid insoluble, DNase and RNase hydrolyzable fractions as described in Methods * Figures average of four parallels

A) (16) with ^3H-thymine uniformly labelled, whereas 69 per cent became acid soluble after ribonuclease treatment Under identical conditions 38 per cent became acid soluble with deoxyribonuclease and 51 per cent became acid-soluble with ribonuclease with ^3H-thymidine-6-T(n).

Additional support for the incorporation of radioactivity from thymine and thymidine into both DNA and RNA fractions was pro-

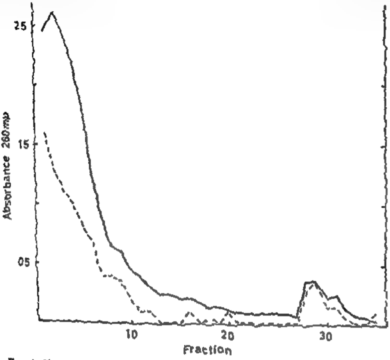


Fig 4 *N meningitidis* cells exposed to ^{14}C-2-thymine Conditions of the experiment as in *Fig 3* (—— = Absorbance, - - = Radioactivity)

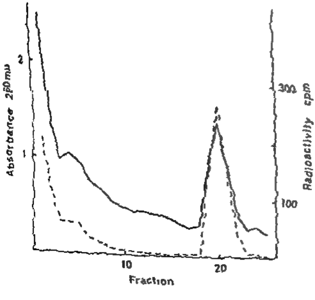


Fig 5 *N meningitidis* cells exposed to thymidine methyl-^3H Conditions of the experiment as in Fig 3) (—— = Absorbance, ---- = Radioactivity)

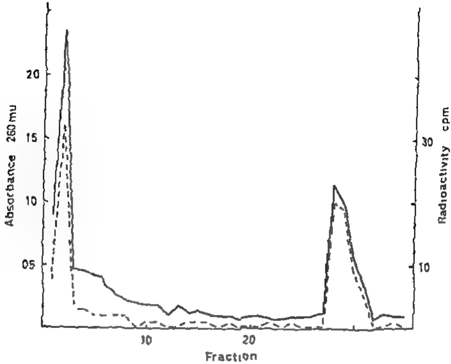


Fig 6 N meningitidis cells exposed to ^{14}C 2–thymidine Conditions of the experiment as in Fig 3 (—— = Absorbance, - -- = Radioactivity)

vided by density gradient centrifugation experiments Radioactivity is found in the peak corresponding to DNA and in the area in which RNA is located after the cells had been exposed to thymine methyl tritium ^{14}C–2–thymine, thymidine methyl tritium or ^{14}C–2–thymidine (Fig 3, 4, 5, 6) When crude extracts were centrifuged the distribution of radioactivity was not significantly different from the distribution of radioactivity from deproteinized and ethanol precipitated samples, only the background radioactivity was somewhat higher The specific labelling of the areas corresponding to the DNA peak and the area in which RNA sediments was tentatively estimated in the way that the nucleic acids were calculated from A_{260} for the first five fractions for RNA and for "total" DNA (Table 4) The labelling of both DNA and RNA is 10–40 times higher

TABLE 4 *Labelling of DNA and RNA in* N meningitidis *from Thymine–methyl ^{3}H, ^{14}C–2–thymine Thymidine methyl–^{3}H and ^{14}C 2 thymidine*

Expt no	Precursor added		Incorporation of ^{3}H and ^{14}C in terms of µg thymine and µg thymidine per µg DNA and µg RNA — DNA	RNA
1	Thymine–methyl–^{3}H	5 3 µg/ml	$7\ 5 \times 10^{-5}$	$4\ 6 \times 10^{-5}$
2	^{14}C–2–thymine	4 1 µg/ml	$3\ 5 \times 10^{-6}$	$2\ 4 \times 10^{-6}$
3	Thymidine–methyl ^{3}H	4 0 µg/ml	$2\ 5 \times 10^{-6}$	$1\ 1 \times 10^{-6}$
4	^{14}C–2–thymidine	7 8 µg/ml	$8\ 3 \times 10^{-6}$	$3\ 9 \times 10^{-6}$

The supplements were added to cultures during logarithmic growth in the KC medium Nucleic acids were extracted and run in a CsCl gradient as described in Methods

with thymine methyl tritium as precursor as compared to the labelling from the other precursors

DISCUSSION

The incorporation of radioactivity from labelled thymine or thymidine into the *N meningitidis* strain M1, calculated in terms of per cent of the material added depends upon the location of the label in the molecule Also, the kinetics of incorporation into the cells is changed when the radioactivity is moved from one part of the donor molecule to another It seems clear that these results represent utilization of break down products of exogenously supplied thymine and thymidine

The experiments also confirm the previous findings (15 16) that the uptake of radioactivity from these compounds is on the whole rather limited The significant difference constantly found between thymine and thymidine probably indicates that thymidine is taken up, or attacked to a lesser extent than thymine The fact that the incorporation from the substrates tested is rather small should be kept in mind when considering the results It could well be, that some of the incorporation observed may be due to impurities in the substrates or to a non specific break down during storage or handling (17, 25)

The way in which thymine and thymidine is broken down and used in the metabolism of *N meningitidis* has not been considered an issue of the present paper However, the experiments indicate that thymine is utilized with fragmentation of the pyrimidine ring with the carbon chain and urea moities catabolically separated (6, 7)

The labelling from the ^{14}C-2-compounds has a pattern which is distinct from those from the differently labelled compounds The incorporation into the acid precipitable material is little influenced by the time Also the uptake at least of ^{14}C-2 thymine is only to a limited extent dependent upon the concentration and the uptake is also to a greater extent susceptible to variations under otherwise identical conditions The findings probably point to a decarboxylation of the C-2, and a reutilization of $^{14}CO_2$ formed (6, 7)

Thymidine phosphorylase EC 2 4 2 4 which is known to catabolize thymidine to thymine and deoxyribose-1 phosphate is not present in *N meningitidis* strain M1 (15) It has previously been suggested that the enzyme thymine-7-hydroxylase which breaks down thymidine by an oxidative attack on, and removal of the 5-methyl group (8 23, 26) might be responsible for the utilization of thymidine in *N meningitidis* (16) This does not seem to be the case, since both ^{14}C-2-thymidine and thymidine-methyl-^3H label RNA as well as DNA such as shown in the nucleic acids separated by density gradient centrifugation (5, 14)

Both RNA and DNA are labelled from radioactive thymine and thymidine This confirms the previous assumption that these compounds can not be used for labelling of DNA in *N meningitidis* under the conditions tested (15, 16)

The ratio labelled RNA labelled DNA was constantly less than the actual ratio RNA DNA Also the labelling obtained in DNA was higher regardless of the location of the radioactivity in the donor molecule Whether this indicates an element of specificity is not known If the findings represent a specific incorporation into DNA, it must probably be by some other mechanism than a thymidine kinase in the way it has been discussed in connection with thymidine uptake in plants and mammalian cells (15)

The preferential labelling of DNA does not seem at least solely to be due to the faint labelling of TMP TDP and TTP from thymidine observed (15) since thymine as well as thymidine in the present experiments give a higher specific labelling of DNA than RNA.

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BRIEF REPORT

A NEW ANTIGEN COMPLEX CO OCCURRING WITH AUSTRALIA ANTIGEN

Lars O Magnius and *Ake Espmark*

Antigenic heterogeneity of Au antigen has been repeatedly described (4–6, 8, 11) Spur formation in immunodiffusion tests has made possible the distinction of several antigenic determinants in the Au antigen complex i e a d, y and x according to Le Bouvier (5 6) The a determinant is considered a common determinant (4 6, 8) The d and y determinants seem to be mutually exclusive (5 6) Au antigen of epidemiologically related cases of serum hepatitis carries either one of these two determinants (6) The x determinant of *Le Bouvier* might be host specified (6)

In this study new antigens co occurring with, but distinct from Au antigen are described and the distribution of one of these the *e* antigen in different categories of Au positive individuals is reported

Material and Methods

Reagents One antiserum was derived from a multiply transfused patient with haemophilia This antiserum has been tested by Dr *B S Blumberg* Philadelphia and found to carry antibodies against Au antigen Another antiserum giving a reaction of identity with the above antiserum was derived from a hospital nurse with no history of previous hepatitis but with continuous exposure to serum hepatitis patients This antiserum was kindly tested by Dr *G L Le Bouvier* New Haven and found to contain antibodies against his *a* and *y* determinants (7) An anti *d* serum was obtained by partial absorption of a high titre human Au anti serum with an *ay* antigen tested by Dr *Le Bouvier*

An antiserum against *e* was derived from an asymptomatic blood donor with no history of hepatitis. This blood donor besides carrying anti *e* was a persistent carrier of Au antigen of the *ad* specificity

A serum from a dialysis patient, a persistent carrier of Au antigen of high titre with the specificity *ay*, was tested by Dr *Le Bouvier* A later serum from this patient was used as a standard antigen during this study

Another serum derived from a patient with hepatitis, besides carrying Au antigen of the specificity *ad*, also contained the *e* antigen. This serum was selected because it gave a strong precipitate with the previously mentioned serum containing antibodies against *e*

Patients Consecutive series of 23 Au positive haemodialysis patients, 43 cases of Au positive hepatitis and 17 Au positive blood donors were investigated Two control groups of Au negative individuals consisted of 151 blood donors and 24 haemodialysis patients respectively

Immunodiffusion tests Sera were tested with the mentioned reagents by the double immunodiffusion technique of *Ouchterlony* The gel consisted of 0 9 per cent agarose (L'Industrie Biologique Française, S A, Gennevilliers, Seine, France) dissolved in 0 01 M HCl tris buffer pH 7 6 Further details of the method have been given elsewhere (10)

Results

It was noticed that sera containing Au antigen of the *ad* specificity often gave single or multiple precipitates with other Au positive sera (Fig 1) These precipitates showed non identity reactions with the Au precipitate indicating that they represented other antigens distinct from Au antigen One of these antigens has been tentatively designated *e* Precipitates of this type were particularly common between sera of Au positive dialysis patients and sera of Au positive blood donors It was shown that the antibody component in this new antigen-antibody system was carried by the blood donors (10)

In testing Au positive sera in the *e* system precipitates indicating antibodies that did *not give* reaction of identity with the *e* precipitate were often encountered, hence there are additional specificities requiring further investigation However, in testing a panel of Au positive sera the same sera

Received 24 1 72 *from the Department of Virology Statens Bakteriologiska Laboratorium 105 21 Stockholm Sweden*

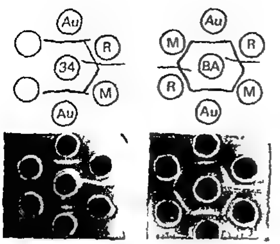

Fig 1 Left Precipitate between two Au antigen R and M both of *ad* specificity where M carries antibodies against the new antigen complex The standard antigen (Au) is of the specificity *ay* and the antiserum (34) carries antibody against the *a* and *y* determinants hence the reaction of partial identity
Right Identity reaction between the *ay* and *ad* antigens when tested against the antibody (B A) Note the reaction of non identity between the Au precipitate and the precipitate between the two *ad* antigens R and M

were detected as positive whether anti *e* or these other antibodies not giving reaction of identity with the *e* system were used Thus there seems to be an association between the presence of these not yet defined antigens and the *e* antigen Presumably, the distribution of the *e* antigen could therefore represent the distribution of all the new antigenic specificities

In these preliminary studies interesting differences in specificity of different categories of Au antigen positive individuals were observed (Table 1) The *e* antigen or the corresponding antibody was not found in any of the sera from the two control groups

Discussion

The new antigen designated *e* described in this report is strikingly associated with Au-antigen and is apparently absent in the normal population With this pattern of distribution this antigen can not easily be fitted into any of the hitherto described iso antigen systems It is also unlikely that the *e* antigen is a constituent of normal liver cells since persistent carriers of Au antigen among dialysis patients carry the *e* antigen to a higher extent than hepatitis patients In hepatitis patients the hepatic injury is more prominent than in dialysis patients as reflected by the elevation of transaminases Hence it is likely that the *e* antigen is determined by the serum hepatitis agent (SH agent) or produced as a host response to this agent

The persistence of Au-antigen in dialysis patients is considered to be due to a deficient immunoresponse of these patients (9) The fact that these individuals in a high frequency carry the SH agent with its associated here described new antigenic specificities is also probably part of the same deficiency However the difference between hepatitis cases and haemodialysis patients with respect to the occurrence of the *e* antigen could at least partially be quantitative rather than qualitative

In asymptomatic carriers among blood donors a postulated tolerance for some essential part of the SH agent may account for the persistence of Au antigen In these carriers weak antigens as e g *e* antigen for which tolerance has not deve

TABLE 1 *Distribution of Specificities in Au Positive Sera from Different Categories*

Category of Au positive individuals	Total number of individuals	Number of individuals with indicated antigen or antibody specificity				Antibodies against the new antigen complex including anti *e*
		y	*d*	*e*	Anti *e*	
Dialysis patients	23	20	3	18	0	0
Hepatitis patients	43	38	5	6	1	1
Blood donors	17	1	16	0	5	13

loped, may exert antigenic stimulation of sufficient duration for detectable levels of precipitating antibodies to be produced

It is tempting to relate the frequent occurrence of antibodies against the new antigens in Au-positive blood donors to recent reports (2, 12) on the low infectiousness of some blood units from persistent carriers of Au antigen. Persistent carriers of Au antigen among dialysis patients are highly contagious (1, 3). These patients carry *e* antigen in a high frequency. The possibility of an association between this antigen and contagiousness is suggested by these findings although further studies are needed before this can be finally settled

Summary

A new antigen complex in Australia antigen positive sera has been detected by immunodiffusion tests. These antigens, one of which tentatively is designated *e*, are distinct from the *a*, *d*, *y* and *x* determinants of the Au antigen complex according to the nomenclature of *Le Bouvier*. The *e* antigen was found in 18 out of 23 persistent carriers of Au antigen from haemodialysis units, in 6 out of 43 Au positive hepatitis cases but in none of 17 Au antigen carrying blood donors. In the latter group however 13 carried antibodies against the new antigen complex, 5 of which were against the *e* antigen. Some evidences are presented for the possible association of this antigen complex in contagiousness

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TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

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Abstracts

Jonas Jonsson & Astrid Fagraeus, Department of Immunologi, National Bact Lab and Department of Immunology, Karolinska Institutet, Stockholm, Sweden POTENTIATION OF THE ORGAN SPECIFIC AND NON ORGAN SPECIFIC SURFACE ANTIGENS OF THYROID CELLS BY STIMULATION WITH PHYTOHAEMAGGLUTININ (PHA)

Phytohaemagglutinin (PHA) from the bean phaseolus vulgaris agglutinates erythrocytes and induces transformation to blast forms in lymphoid cells It also induces mitotic activity in lymphoid and some non lymphoid cell types e g amoebae human epidermal and rat fibrosarcoma cells PHA may also induce the production of immunoglobulin in lymphoid cells This is a report of an increase of thyroid cell surface antigens caused by stimulation with PHA

The antigenic properties of thyroid cells obtained at surgery were examined by the mixed haemadsorption technique (2) and the indirect immunofluorescence technique (3) using monolayer cultures and suspended cells from monolayer cultures Two organ specific human anti thyroid sera, one producing ring and the other filled zones in the mixed haemadsorption test (4), one non-organ specific homologous serum and one monkey anti human serum were the principal reagent sera.

The organ specific reactivity of primary cultures decreased from the third day of growth and were usually not demonstrated after 7 days Incubation of the culture with PHA P (Difco Laboratories) 100–500 μg/ml culture medium within 5 days after the organ specific reactivity had disappeared caused it to reappear

Secondary cultures usually had no organ specific antigens and the non organ specific ones were decreased Both types of antigens reappeared partly after incubation with PHA

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BY SERUM ANTIBODIES AS WELL AS LOCAL ANTIBODIES

Employing the ileal loop technique, the resistance to challenge with cholera enterotoxin was compared between unimmunized rabbits and rabbits immunized with toxin of toxoids It was shown that subcutaneous as well as intraintestinal immunization induced protective immunity the toxin being a better immunogen than formalin induced toxoid and much better than heat induced toxoid

The relation between protection and serum antitoxin titre was poor e g protection was seen in the abcence of demonstrable serum antibodies However intravenous administration of antitoxic antiserum conferred some protection suggesting that local as well as serum mediated antitoxic immunity is operating in the host defence against cholera

Ann Mari Svennerholm J Holmgren & O Ouchterlony, Institute of Medical Microbiology University of Goteborg Goteborg Sweden ANTIBODIES TO A BACTERIAL ENDOTOXIN MEASURED BY INDIRECT HAEMAGGLUTINATION AND BACTERICIDAL TESTS—DIFFERENCES IN IMMUNOGLOBULIN CLASSES AND SPECIFICITY

In a study of the antibody response in rabbits to O antigen from *Vibrio cholerae* it was found that measured with indirect haemagglutination IgM antibodies predominated in the primary as well as in the secondary response In contrast when

measured with a bactericidal technique, O anti bodies of the IgG class were generally found in titres equal to the IgM titres during the primary response and during the secondary response the IgG antibodies dominated

The differences could be ascribed to a superior detection of IgG antibodies in the bactericidal as compared to the indirect haemagglutination method, while the techniques were about equally apt for detection of IgM antibodies In addition, absorption experiments indicated that minor determinants on the endotoxin participating in the bactericidal reaction might be blocked by the attachment of the antigen to the erythrocytes in the indirect haemagglutination test

Peter Larsson, Lars Åke Hanson Ulf Jodal and Knut Lincoln, Institute of Medical Microbiology and Department of Pediatrics, University of Goteborg, Goteborg, Sweden STUDIES OF PROTEUS BACTERI CAUSING URINARY TRACT INFECTIONS (UTI)

Proteus mirabilis and vulgaris strains from geriatric patients and from children have been classified into the O groups of Kauffmann and Perch Anti sera against 40 of the 49 known O groups were used for testing Boiled bacterial cultures were incubated with antiserum at 50° C and agglutination was read In children also antibody titres against the infecting strains were determined by indirect haemagglutination

Results 197 cultures from 126 children and 120 cultures from 33 geriatric patients were tested, of which 60 and 80 per cent respectively could be typed The most common O groups were 3, 10, 23, 27, 28, 30 A few strains giving spontaneous agglutination were labelled rough

Records from 99 children showed that 23 had myelomeningocele with UTI, 31 cystitis 28 asymptomatic bacteriuria and 6 pyelonephritis (two with a definite diagnosis) No significant difference in O groups could be found between the patient groups

Antibody titres against 11 pooled Proteus antigens were $^1/_2$–$^1/_{32}$ in healthy persons and in cases of cystitis There was no titre rise during the cystitis One patient with suspected pyelonephritis showed a titre rise from $^1/_{16}$ to maximally $^1/_{512}$ and developed mercaptoethanol resistant antibodies. Another patient with pyelonephritis had repeated infections with an O3 strain and titres of $^1/_{[illegible]}$ consistantly reduction resistant

The geriatric patients had titres of $^1/_{16}$–$^1/_{128}$ with some having higher, maximally $^1/_{512}$.

S Olling, A Brandberg, Lars Åke Hanson, J Holmgren, U Jodal & K Lincoln, Institute of Medical Microbiology and Department of Pediatrics, University of Goteborg, Göteborg, Sweden THE RESISTANCE TO THE BACTERICIDAL ACTIVITY OF NORMAL HUMAN SERUM AMONG E COLI STRAINS CAUSING URINARY TRACT INFECTIONS

Altogether 600 strains of *E coli*, isolated from faeces of healthy individuals and from urine of patients with urinary tract infections were studied with regard to their sensitivity to the bactericidal activity of normal human serum The per cent surviving bacteria after incubation with each of five different blood donor sera was determined (Olling *et al*, to be published)

The results obtained with the five blood donor sera were similar

Analysis of bacteria from faeces and urine of O groups commonly found in strains from these locales showed that they were more resistant to the serum bactericidal activity than were bacteria of less common O groups There was no relation between these findings and the antibody titres to different O antigens in the employed sera

Rough *E coli* strains isolated from faeces and urine were very sensitive to the bactericidal activity

Strains from children with asymptomatic bacteriuria were significantly more sensitive than strains causing symptomatic infections and strains from faeces The possible implications of these findings for the appearance and course of urinary tract infections will have to be further investigated

B Kaijser, John Erik Brorson, Lars Åke Hanson & S Seeberg, Institute of Medical Microbiology and Institute of Pediatrics University of Goteborg Goteborg, Sweden IMMUNOLOGICAL STUDIES OF THE L FORM OF SOME ESCHERICHIA COLI STRAINS

The L form of some Enterobacteriaceae species is by many authors proposed to cause urinary tract infection As they are less sensitive to some antibiotics than their corresponding bacteria they might survive a period of antibiotic treatment Their later convertion into bacteria might cause a relapse As a basis for studies concerning the role of L forms from *E coli* in urinary tract infections, we have studied their immunological characteristics

Methods The L forms were induced by penicillin from three *E coli* strains (serotypes 08 K8 H4, O2 K1 H4, O15 K? H⁻) They grew on solid media with the typical "L-colonies" and could be subculuvated They did not grow on ordinary media for *E coli* cultivation Stained films from "L-colonies" showed gram negative, spherical or pleomorphic microorganisms Washed

and formalin killed L forms that were subcultivated at least four times were used for immunization of rabbits The following techniques were used for the studies double diffusion in agar, indirect haem agglutination, single radial immunodiffusion and immunofluorescence

Results In all the abovementioned *L forms*, O antigen could be shown *by using double-diffusion* in agar as well as immunofluorescence and haemag glutination Although the amount of O antigen was less in the L forms than in the whole bacteria it was sufficient to induce a marked O antibody response in the immunized rabbits In the L form of *E coli* O8 K8 H4 an antigen with the charac teristics of K8 was detected Furthermore, in all of the studied *L forms* many of the other antigens from the whole bacteria, were found Among these was a protein antigen identified in all of 29 dif ferent studied *E coli* strains and in some Proteus and Pseudomonas strains

Conclusion Many of the antigens from three studied *E coli* strains were also found in their corresponding L form O antigen sufficient for in ducing an antibody response when injected in rab bits was found in all the L forms Thus, there are possibilities for an antibody response to E coli O and K antigen in urinary tract infections caused by L forms

Monica Grandien and A Espmark, Department of Virology, Statens Bakteriologiska Laboratorium Stockholm ANTIBODY RESPONSE TO RA BIES IMMUNIZATION AS DETERMINED BY INDIRECT IMMUNOFLUORESCENCE

Antibody responses to post exposure rabies vaccina [illegible] various (IF) [illegible] mouse brains was used Titres were derived from 3 fold dilution series of test sera

In 6 cases acute sera and 7 10 days sera were available all acute sera were negative whereas the latter including those drawn on day *7, 8* and *9* were positive with moderate titres Two further vaccinees without acute sera were antibody positive in their first sample taken on day 5 and 18 re spectively All these [illegible] patients were bled 4 days after termination of the vaccination course and exhibited IF titres of 27–243 An inadequately immunized patient had an antibody response de layed until day 26 Five further vaccinees were bled *only after completion of the vaccination* course (on day 18 to 47) They were all antibody positive (titres 27–243)

Four patients were followed for about one year One of them, having received booster doses at 3½ months, showed a titre of 27 at the control after one year The other three had titres of 3, 9 and 9 respectively

Experiments have been initiated to study the *correlation between* immunofluorescence titres and neutralizing titres and *protection*

Brith Christensson, A Espmark and H L Kott meter, Statens Bakteriologiska Laboratorium and Radiumhemmet Stockholm ANTIBODIES TO HERPES SIMPLEX VIRAL ANTIGENS AND CELL MEMBRANE ANTIGENS IN PA TIENTS WITH CERVICAL CARCINOMA

Sera were collected at Radiumhemmet, Stockholm from 60 women with carcinoma of the cervix uteri in the active stage and from patients treated for this disease within the preceding 12 months Con trol groups consisted of 60 age matched healthy *women and a group of 40* patients *with recurrent* Herpes simplex virus (HSV) infection Sera were titered for HSV neutralizing antibodies and for antibodies to surface antigens in HSV infected cell cultures and in certain permanent human tu [illegible]

mixed haemadsorption (MH) technique was em ployed

The mean neutralization antibody titre by con ventional tests was 89 in the carcinoma group and 25 and 28 respectively in the two control groups In the 12 patients which had been treated 6–12 months earlier the mean antibody titre in creased from 100 to 316 after the operation

Kinetic neutralization tests showed that 78 per cent of the cervix carcinoma group and 15 per cent of the control groups had K values of 4 or higher against type 2

By the mixed haemadsorption test 88 per cent of the carcinoma group exhibited antibodies to cell surface located antigens of HSV infected GMK or RK 13 cell cultures Corresponding figures for control groups were [illegible] per cent and 13 per cent respectively There was no obvious correlation be tween neutralizing titres and mixed haemadsorp tion titres

Reactivity to surface antigens of non infected HeLa HEp 2 and Chang liver cells was more com mon in the carcinoma group sera (≈ 40 per cent) than in the control groups (2 5 per cent) as shown by MH tests

L Magnius, A Espmark & O Ringertz, Depart ments of Virology and Bacteriology Statens Bak [illegible] Stockholm Sweden [illegible] OF AU [illegible] ENT AU [illegible]

Using the Ouchterlony technique spur formation of immunoprecipitates between *Australia-antigen-po* sitive sera and certain antisera has been observed

by several authors (Levene, Kim, Le Bouvier and others) In addition to the common determinant *a* Le Bouvier has also identified the determinants *d* and y, which seem to be mutually exclusive

In the present study, human antisera with different antibody specificities have been used and spur formation has been considered an indication of antigenic heterogeneity In a consecutive series of 23 Au positive dialysis patients 20 carried Au antigen of the *ay* type Also in a series of Au positive hepatitis cases the majority, 38 out of 43, carried Au antigen of *ay* type In persistent Au-carriers among blood donors *ad* antigen occuring in 16 out of 17 was more common than *ay* antigen In 16 examined Au-positive sera collected 1961 and 1962 from an outbreak of the so called "track finders hepatitis" the Au antigen was of *ad* type In 1971 sera were collected from 438 track finders with previous hepatitis during the outbreak 1959 to 1962 Four of these were still carrying Au antigen i e in a frequency ten times higher than that in the normal population These four were of *ad* type

Thus the Au antigen found in the track finders was of the type less commonly encountered in cases of clinical serum hepatitis in Sweden This favours the assumption of epidemiological unity in the outbreak of track finders hepatitis

U Forsum & A Forsgren, Institute of Medical Microbiology University of Uppsala, Uppsala Sweden PROTEIN A FROM STAPHYLOCOCCUS AUREUS AND NONSPECIFIC REACTIONS IN IMMUNOLOGICAL DIAGNOSTIC TECHNIQUES

The fact that difficulties are involved in the reproducibility of serotyping of *S aureus* is well known Conflicting results have been published concerning the agglutination of *S aureus* in rabbit sera However, a reaction between protein A of *S aureus* and the Fc part of the antibodies used for serotyping obviously can contribute to the difficulties 87 of 137 *S aureus* strains agglutinated in normal rabbit serum The agglutination was shown to be caused by the Fc part of IgG F(ab^1)$_2$ fragments of IgG or intact IgM in corresponding concentrations were *unreactive* The agglutinating strains had high or moderate contents of protein A Strains with low contents of protein A and protein A negative mutants *did not* agglutinate The importance of the reaction between the Fc part of IgG and protein A for serotyping of *S aureus* is demonstrated Adsorption of immune serum with *S aureus* according to a standard method (Oeding) for production of specific factor sera for serotyping of *S aureus* completely removes IgG but not IgM Two alternative methods for serotyping of *S aureus* are suggested, using either F(ab^1)$_2$-fragments of IgG or intact IgM

A Forsgren, Institute of Medical Microbiology, University of Uppsala, Uppsala, Sweden PATHOGENICITY TESTS WITH PROTEIN A MUTANTS OF STAPHYLOCOCCUS AUREUS

As a consequence of its reaction with the Fc region of γG globulin, protein A from Staphylococcus aureus elicits hypersensitivity reactions and has an anti phagocytic effect The primary purpose of the present work was to investigate the role of protein A in staphylococcal infections

After treatment with ethylmethanesulphonate or nitrosoguanidine, mutants of *S aureus* deficient in protein A, and mutants with various combinations of deficiencies in protein A, nuclease, coagulase, α haemolysin, fibrinolysin, mannitol utilization, and the phage type pattern were isolated

The pathogenicity for mice of *S aureus*, Cowan I and derived mutants and revertants was investigated by two methods one involving intravenous injections and the other, subcutaneous injections in cotton dust Mutants and revertants with reduced production of protein A and α haemolysin or loss of protein A and coagulase showed only a slight decrease in pathogenicity as compared with the wild type, as determined by the two pathogenicity tests Mutants which lacked protein A, nuclease, coagulase, fibrinolysin, mannitol utilization, and phage type pattern, and mutants producing only nuclease or α haemolysin showed significantly decreased pathogenicity Reproducible results were obtained with both subcutaneous and intravenous injections No spontaneous reversion was detected *in vivo* It is concluded that the pathogenicity of *S aureus* depends on the combined effects of many products including protein A

B Nystrom, Department of Clinical Microbiology, Karolinska sjukhuset, Stockholm Sweden INACTIVATION OF B SUBTILIS SPORES WITH SUBATMOSPHERIC STEAM AND GASEOUS FORMALDEHYDE

Sterilization with subatmospheric steam and formaldehyde was first described by Alder An autoclave according to this principle is manufactured in Sweden by AB CTC The inactivation of B subtilis spores has been studied with varying sterilization cycles

After preheating with low temperature steam and a pre vacuum, gaseous formaldehyde is in

then pressure and temperature pulsate with an amplitude of around 100 millibars After a post vacuum period air is introduced

As test pieces have been used B subtilis spores,

dried together with quartz sand and NaCl crystals, described and manufactured by Christensen The inactivation factor has been determined as the ratio between the number of viable spores in the untreated test piece and the number of viable spores in the test piece after sterilization

At 78° C and with 15 ml of gaseous formaldehyde in the 100 litre autoclave chamber, FH-period 2 min and FSH period 4 min, the inactivation factor is 2 2 × 105 Lengthening of any of the periods gives significantly lower inactivation factors A temperature change of ten centigrades changes the inactivation factor about tenfold

Further investigations are planned to study the effect of various amounts of gaseous formaldehyde, and to study the inactivation factor of an optimal sterilization cycle for other spores as well as for various viruses, mycobacteria and vegetative bacteriae

Kristina Wickman and Ingrid Blomberg, techn ass From the Department of Clinical Microbiology, Karolinska Sjukhuset Stockholm SENSITIVITY DETERMINATION WITH A NEW ANTIMYCOTIC AGENT, BAY B 5097

Bay b 5097 is the preliminary name of a trityl imidazol derivate with a broad fungistatic activity, synthethized by Bayer Germany It has been used in Sweden in about 20 cases of verified Candida septicaemia with rather promising results We therefore thought it valuable with a simple method for sensitivity determination, preferably one according to the disk diffusion method recommended by WHO for sensitivity testing of bacteria

Bay b 5097 is poorly water soluble so it had to be dissolved in acetone for preparation of the disks Candida species grew readily on Mueller Hinton medium They gave a satisfactory density of colonies after standard dilution and incugation 24 hours at 37° C With 10–20 mcg in the disk most Candida species were inhibited with zone diameters of 30–40 mm Small zones of 10–15 mm were obtained with staphylococci With other tested bacteria no zones appeared

A preliminary regression line was constructed by plotting the zone diameters against the MIC of Bay b 5097 in agar plate dilution series According to this regression line Candida species were sensitive to 0 005–0 25 mcg/ml and staphylococci to 2–16mcg/ml of Bay b 5097

P Wåhlen, John Erik Brorson & V Magursky Dept of Clin Bact, Inst of Med Microbiol, Univ of Goteborg and Dept of Obstet and Gynaec, Lidköping Hospital, Lidköping Sweden THE OCCURRENCE OF YEASTS AND BACTERIA IN THE VAGINA OF GRAVIDAE

Muco-cutaneous yeast infections in new borns have by many investigators been considered to the caused by organisms present in the maternal vagina On account of these proposals a microbiological examination was made of consecutive specimens of vaginal secretions obtained ante partum

One thousand vaginal secretions were examined by direct microscopy of Gram stained smears One hundred of these specimens were also examined by a simple quantitative cultivation method after transportation in transport medium kept in the cold as well as at "room temperature"

On direct microscopy lactobacilli and diphteroid rods were most often recognized These findings were as a rule verified on cultivation of specimens transported in the cold The best agreement of results on this comparison was obtained when the specimens contained considerable amounts of yeast (15 of totally 35 positive cultures) Direct microscopy thus proved to be a rather dependable method in the detection of vaginal yeast

Cultivation of the portions of specimen duplicates which were transported at "room temperature" (12–36 h) gave results extremely different from those of cooled portions Especially coliforms and enterococci showed a great tendency to multiply, while the number of alpha streptococci and diphteroids greatly diminished These findings certainly holds true for many other types of transport medium specimens

Kristina Wickman & S O Liljedahl, Department of Clinical Microbiology and the Burns Unit [illegible] Sweden THE [illegible] WITH [illegible] ORA [illegible] AND INFECTION RATE OF SEVERE BURNS

During the years 1962 1966 the bacteriology of 308 patients treated in the Burns Unit was studied In 1967 treatment with warm dry air was introduced Before the end of 1970 about 100 patients had been treated

A general comparison between the two periods showed that the number of very extensive burns had increased The mean calculated mortality risk (based on age and per cent of burned body area) was more than 98 per cent, which means that virtually only patients with 100 per cent mortality

risk were lost In spite of this, the bacterial flora of the wounds was much alike in the two periods, both qualitatively and quantitatively

By comparison of the infection rate of 45 treated patients with that of 45 non treated with the same mortality risk, we found about the same frequency of septicaemia But while all the non treated patients died, more than half of the air treated survived Among the 200 patients of the period only five died of infections, all of them with 100 per cent mortality risk

The results—about the same bacterial findings but a decrease of severe systemic infections—could be explained by the dry air creating unfavourable surroundings especially for the gram negative bacteria

B Wretlind, L. Hedén, R Möllby & T Wadström, Department of Clinical Microbiology, Karolinska Sjukhuset and Department of Bacteriology Karolinska Institutet, Stockholm, Sweden HAEMOLYSINS AND OTHER LETHAL PROTEINS FROM AEROMONAS HYDROPHILA AND PSEUDOMONAS AERUGINOSA

Many gram negative rods, especially among the related genera *Pseudomonas*, *Aeromonas*, and *Vibrio*, produce extracellular protein toxins but very little is known about their role in infection *Pseudomonas aeruginosa* produces lethal toxins, such as phospholipase,, proteinases, one of which has elastase activity, and a lethal protein without known enzymatic activity *Aeromonas hydrophila* produces a lethal haemolysin and possibly also another lethal protein *A hydrophila* strain B3646 produces extracellular haemolysin in different defined and complex media The haemolysin is released from the cells in the late stationary phase together with a proteinase and malate dehydrogenase Addition of chloramphenicol in the early stationary phase does not inhibit the release This indicates that the haemolysin is formed intracellularly during the logarithmic phase of growth and is later released into the medium by autolysis The haemolysin has been partially purified by iso-electric focusing It is separable into two components with iso-electric points of 3 5 and 5 5 Most strains of *Pseudomonas aeruginosa* are haemolytic on blood agar plates However, haemolysis is not released from the cells grown in liquid media but from the cells grown on dialysis membranes on complex solid media The haemolysin stands boiling and is resistant to five different proteinases It is probably not related to the haemolysin produced by *A hydrophila*

Dan Danielsson, G Laurell and S Öhman, Dept of Clin Bact, Central County Hospital, Orebro, and the Inst of Med Microbiol & Dept of Dermatology, University of Uppsala, Uppsala, Sweden CULTURE DIAGNOSIS OF GONORRHOEA—A COMPARISON OF THE RESULTS OBTAINED BY CULTURE IN THE CLINIC, AFTER TRANSPORT IN STUART TRANSPORT MEDIUM AND WITH THE USE OF TRANSGROW

Specimens from 91 patients, 59 males and 32 females, who attended the outpatient clinic for venereal diseases in Uppsala, Sweden, were examined with regard to *Neisseria gonorrhoeae* in the following way 1) by transport (16–22 hours) and growth on Transgrow (TG) medium (Martin & Lester, HSMHA Health Reports, *86* 30–33, 1971), 2) by culture on selective and non selective gonococcal (GC) medium a) in the clinic, b) after transport in modified Stuart transport medium (STM) for 2–4 hours, c) after transport in STM for 16–22 hours The highest yield of GC isolates was obtained by culture in the clinic, a diagnosis of gonorrhoea being arrived at in 57 patients, 42 males and 15 females Correspondingly, culture after transport in STM gave a positive diagnosis in 56 patients, 42 males and 14 females after transport for 2–4 hours, and in 39 males and 17 females after transport for 16–22 hours, Transport and growth in TG medium gave a positive diagnosis in 52 patients, 35 males and 17 females Four patients harboured gonococci that did not grow on selective medium, and consequently, they were negative on TG By combining the results of the four procedures (1 and 2a, b & c) a diagnosis of gonorrhoea was arrived at in 64 patients in all This means that a diagnosis of gonorrhoea was lost in 11 per cent of the patients by culture in the clinic, in 12 5 per cent after transport in STM and in 19 per cent after transport and growth in TG

P Wåhlén, Dept of Clin Bact, Inst. of Med Microbiol, Univ of Goteborg, Sweden DECONTAMINATION OF SPUTUM SPECIMENS WITH THE SODIUM LAURYL SULPHATE METHOD

The method most used in Sweden for mycobacteriological examination of sputum implies decontamination with 4 per cent NaOH (equal vol), cultivation on L-J medium and guinea pig test The guinea pig test is very sensitive but has disadvantages from economic and laboratory safety points of view Exclusion of the guinea pig test with maintenance of a reasonably good standard of the examination presupposes the use of a more effective and less harmful method of decontamination One such method was tested

One thousand samples of sputum were each split into two equal parts One part was examined according to the conventional method including cultivation and guinea pig test The other part was decontaminated according to the Na lauryl sulphate method (Tacquet & Tison, modif Engbaek) and only cultivated

The Na lauryl sulphate method gave considerably fewer contaminated cultures (0 8 per cent as compared to 6 0 per cent) In addition ,this method gave a few more positives (8 9 per cent) than did the other routine including guinea pig test (8 1 per cent)

With the introduction of the Na lauryl sulphate method for decontamination there is no longer reason to maintain the guinea-pig test for mycobacteriological examination of sputum

Anna Stina Malmborg, Siv Seim & Bengt Wretlind, Department of Clinical Microbiology, Karolinska Hospital, Stockholm IDENTIFICATION OF ENTEROBACTERIACAE WITH THE API SYSTEM

The API system (Analytab Products, Inc) is a multitest, micromethod system for identification of Enterobacteriacae Each series in the system concists of 10 or 20 biochemical tests The substrates are kept in sterile plastic tubes A single colony of the bacterial strain is emulsified in distilled water (4 ml), and inoculated with a Pasteur pipette into each tube The series is incubated over night at + 37° C

In the present study, the API system was compared to conventional tests The bacteria used were 16 stock cultures, and 79 clinical isolates of gram negative rods

There was a good agreement between the results of API tests and conventional tests Of the 95 strains tested, 87 (92 per cent) were correctly grouped with the API system One strain was in correc [illegible] tified [illegible] groupe [illegible] fied as ONPG negative E coli with the API system One strain of Salmonella gave results in the API series only suggestive of a Salmonella strain

The principal disadventage of the API system is the long time required for inoculation The principal advantages are minimal storage space required, readiness for use stability for one year at + 4° C, and accuracy of identification of Enterobacteriacae within 24 hours

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REPRODUCIBILITY IN BACTERIOPHAGE SENSITIVITY PATTERN OF *PSEUDOMONAS AERUGINOSA*

TOM BERGAN and ARVE LASTAD

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The reproducibility of phage sensitivity patterns of *Pseudomonas aeruginosa* was studied by (i) duplicate typing of the same strains (ii) by comparing the recordings made by two independent observers, and (iii) by letting both observers read all plates twice Serial cultures from the same patients were also studied The results indicate that differences in one and occasionally in two reactions are consistent with a common origin of strains

In a previous article, a new bacteriophage typing set for *Pseudomonas aeruginosa* has been presented (2) The precision with which sources of infection and paths of transmission may be elucidated will depend on the stability of the phage typing pattern

It is the purpose of this communication to analyze the reproducibility of the phage sensitivity pattern of *P aeruginosa*

MATERIALS AND METHODS

Bacteriophages and Bacteria

The typing set consisted of the phages 73, F7, M6 Me13, 113 F116, Px3, C1c, C4, C13, C21, H249 P10 VII XVI, Z2, Z3, Z19, and Z20 The 502 pseudomonas strains typed are listed in Table 1

Bacteriological Procedures

The typing technique and the culture media used have been detailed previously (2)

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Plan of Study

In order to evaluate objectively the stability of the phage sensitivity pattern, the 502 pseudomonas strains were tested twice, on different days All plates were read independently by two observers to obtain an estimate for the difference in reading of two individuals Each observer studied every plate twice to yield a measure for the intra-observer error Due to the number of plates read per day, all readings were unbiased from previous knowledge of phage pattern Before the study started, for a training, the observers jointly evaluated a number of plates The stability patterns of sequential cultures from the same patients were also studied

Statistical Evaluation

The differences between observer discrepancies were tested by a Bernoulli model by the procedure shown by *Weber* (7)

RESULTS

Intra observer Discrepancy

Each plate being typed twice, a total of 1004 plates were observed The discrepancy made by each individual observer is discernible from Table 2 It is seen that the two observers read the plates with different precision, the variation was less for observer A Coun-

ting only plates with differences in more than one + or + + reaction (designations used as in reference (1)), observer A recorded different phage patterns for 2 per cent and observer B for 4 per cent of the plates The difference in observer discrepancies, however, was not statistically significant ($P > 0.05$) The bacteriophages with the most variation were *Px3* and *H249* – When plates were read twice, the non-typable strains were reduced by 1–2 per cent

TABLE 1 *Presentation of Strains of* Pseudomonas aeruginosa *Employed*

Number	Source
86	Phage propagating strains (see Table 1, (1))
12	Dr B W Holloway, School of Microbiology, University of Melbourne, Parkville, Victoria, Australia
13	Dr E J L Lowbury, Burns Unit, MRC Research Unit, Birmingham Accident Hospital, Birmingham, U K

TABLE 1 *continued*

Number	Source
8	Dr R G Doggett, Texas Institute for Rehabilitation and Research, Texas Medical Center, Houston, Texas, USA
1	NCTC 6749
1	Microbiological Department Haukeland sykehus, Bergen, Norway
11	Dr M T Parker, Cross Infection Reference Laboratory, Colindale, London U K
14	Dr J Govan, University of Edinburgh, U K
26	Dr B Thom, Cross Infection Reference Laboratory, Colindale, London, U K
23	Dr B Lányi, National Institute of Public Health, Budapest, Hungary
1	Dr O Sandvik, Veterinary College of Norway, Oslo, Norway
24	Various serogroup reference strains presented previously (1)
282	Strains from diagnostic specimens at the Rikshospitalet Oslo, Norway
502	Total

TABLE 2 *Results of Independent Double Reading by Two Observers after Two Bac Recorded Sens*

Row	Class of discrepancy	*No of comparisons*	Total no of plates with differences	No of plates with differences in recorded reactions: 1	2	3	4	5	≧6	Sum ≧2	Total of reacl differer
A	*Intra observer* discrepancy for person A	*1004*	*105*	*84*	*17*	*2*	*1*	*1*		21	133
B	Intra observer discrepancy for person B	1004	204	116	21	10	3	4		38	*220*
C	Inter observer discrepancy	1004	186	144	32	[illegible]	2	3		42	246
D	Differences in two typings as *recorded by* observer A	502	201	114	36	12	4	1	4	57	314

Intra observer discrepancy has been noted when a + or a + + reaction (designations used in the same sense as in reference (1)) was recorded once and a negative reaction at the other reading

In noting *inter observer discrepancy* , two recordings of each observer were evaluated Observer inconsistencies make several situations possible The evaluation system is explained on the hand of the following example

A_1	73,	F7	M6	Me13	113	F116, (Px3)
A_2	73,	(F7)			113	F116 (Px3)
B_1	73,			Me13		
B_2	73,				(113)	F116

Inter observer Discrepancy

The discrepancy between the two observers (Table 2) was slightly higher, but not significantly different from the intra-observer discrepancy (P>0 05) Fortytwo plates among the 1004 had two or more differences in + or ++ reactions

Differences in Phage Sensitivity Patterns between Two Typings

For the purpose of these evaluations the records of observer A who exhibited the best precision in reading were used The differences in phage sensitivity patterns shown when each bacterial strain was typed on two different days appear in Table 2 row D The figures obtained are higher than for the intra observer discrepancy of observer A indicating that the real differences in patterns are considerable since the 57 plates in row D of Table 2 derive from 502 plates (*i e* 11 4 per cent) and the 21 reactions in row A refer to 1004 comparisons (*i e* 2 1 per cent), the pattern differences for some 9 per cent of the plates are ascribable to the phage host systems as such and not to observer discrepancies

Phage Sensitivity Pattern in Sequential Cultures from the Same Patient

Whereas the above data demonstrate the technical variation inherent in pseudomonas phage typing epidemiologically relevant data may be obtained from strains of a common source Probably, no single laboratory investigation can demonstrate the epidemiological relevance of pattern distinction Consequently sets of sequential pseudomonas isolates have been compared The finding on 128 isolates from 42 patients are summarized in Table 3

In an attempt to assess the permissible variation between isolates strains of the same serogroup from each patient were analyzed separately Accordingly 81 isolates from 32

phage Typings of 502 Strains of Pseudomonas aeruginosa The number of Differences in the Patterns are Indicated

No of recorded differences in reactions of each typing bacteriophage																			Non typable at only one of the recordings
73	F7	M6	Me13	113	F116	Px3	C1c	C4	C13	C21	H249	P10	VII	XVI	Z2	Z3	Z19	Z20	
6	3	3	7	11	5	[illegible]	4	4	11	2	[illegible]	1	13	7	7	5	6	3	10
7	7	11	6	13	9	25	16	7	23	7	29	3	21	14	15	1	3	3	16
	11	10	15	13	8	27	13	7	42	5	25	6	22	13	8	6	12	1	11
6	2	13	18	11	14	27	24	8	42	7	35	7	19	13	21	[illegible]	15	5	28

where A and A are the recordings made by observer A and B_1 and B_2 the corresponding recordings made by observer [illegible] Here a difference would be recorded only for phage F7 *i e* a + or a ++ reaction observed only once or a ± reaction (figure in parentheses) is considered to represent an inconsistent reaction which must be interpreted more liberally

TABLE 3 *Summary of Evaluation of Phage Susceptibility Pattern in Sets of Sequential Isolates of* Pseudomonas aeruginosa *from the Same Patient *)*

Row	Specification			Number
1	Sources of isolates	Urine	54	
		Wound/ulcer	31	
		Tracheal/bronchial secretion	25	
		Throat/sputum	7	
		Cyst, ear, eye, pus, unknown	11	
2	No. of isolates examined			128
3	No. of patients with several sequential isolates			42
4	No. of sets (patients) with more strains of the same serogroup			32
5	No. of isolates included in row 4			81
6	Mean no. of cultures tested per set (of row 4)			2.6 (range 2–11)
7	No. of sets (of row 4) with variation			20
8	No. of isolates included in row 7			55
9	Strains (of row 4) with difference in			
	one reaction			8
	two reactions			8 } = 16 } = 24
	three reactions			4
	four or more reactions			4
10	Mean no. of differences per set (of row 4)**)			1.2 (range 1–7)
11	Mean no. of common reactions in sets with variation			3.3 (range 1–10)
12	No. of sets with more than one serogroup occurring			28
13	Mean no. of phage susceptibility differences for isolates of row 13			2.8 (range 0–8)
14	Mean no. of serogroups in sets of row 13			2.3 (range 2–5)
15	Mean observation period (in days) for all 42 patients			49 (range 1–247)

*) The first strain isolated or a strain with the most frequent phage susceptibility pattern was used as index strain for comparisons in assessing differences in phage sensitivity. The recordings of observer A have been employed, i.e. after double reading of strains typed twice. A phage for which a + or a ++ reaction was observed at least twice or a + or ++ reaction once and a ± reaction the second time, was included in the phage sensitivity pattern.

**) The mean variation per set was obtained by counting the number of variations observed within each set of isolates belonging to the major serogroup of each patient. Each such set of the same serogroup was weighted equally regardless of set size. In this connection cases where all strains from a patient belonged to different serogroups were non evaluable.

patients showed a variation in the pseudomonas sets from only 20 patients. Two or more reactions differed in 16 of the 24 possible comparisons resulting. The mean number of differences within sets belonging to the same serotype was 1.2 as contrasted with 2.8 when all sets with more than one pseudomonas serogroup were also included.

DISCUSSION

Variation in phage typing patterns may have many explanations The initial ratio between number of infectious phages and bacterial cells is important In routine phage typing, this multiplicity of infection will vary somewhat Differences in results may be the con sequence of differences in the physiological states of the bacterial strains, the actively multiplying cells being more susceptible Phage host interaction has an optimal tem perature, reduced plaque formation may be found if plates before incubation have been left at room temperature for a longer period than usual The size of the plaques being a relevant factor, a hand lens is required for the study of small plaques The variation caused by the human element is documented in this communication

The independent double reading of two typings per strain have shown that the order of variation for retyping was ca 5 times that of repeated readings of the same plates For a practical application of the procedure, the central question is what degree of varia tion in phage sensitivity pattern may be tolerated between epidemiologically related cul tures When pseudomonas strains were re typed on different days, approximately 60 per cent showed identical pattern reactions If variation in only one phage reaction were tolerated 11 4 per cent of the strains would be regarded as different if retyped Only 4 2 per cent of the strains differed in more than two reactions

This parallels the findings of *Williams & Rippon* (8) with staphylococci Although they defined their typing procedure in detail, they observed considerable differences when the same cultures were retyped Lysis by the same phages was observed in only 46 3 per cent of duplicate typings when multiple plates were made simultaneously from the same broth and in a mere 21 1 per cent when the same strains were typed on different days Assuming identity for the bacterial populations in both typings, *Williams & Rippon* ascribed the difference to technical variation changes arising during storage of cultures, or to the simultaneous existence of two strains within the same culture Such variation has led to abandonment of the original concept that each individual phage pattern represent a separate type (9) Reactions of all strengths must be considered for the comparisons of different strains

Our findings on pattern stability are of the same order as found for pseudomonas by *Sutter et al* (6) For 50 bacterial strains, they found that 70 per cent showed the same pattern upon duplicate typing on the same day and 58 per cent when retyping on successive days They found an increased variation after storage of the bacteria for 1–3 months before retyping but never in more than one reaction Retyping after 4 months storage, *Sjöberg & Lindberg* (5) found that 27 per cent differed in one phage reaction, 6 per cent in two reactions, and 3 per cent in three reactions *Meitert* (3) using a particular code system could observe stable phage patterns after passage of pseudomonas strains in mice After three subcultures and storage on slants for 6–12 months, however, a change in phage type was observed in 16 per cent (3) *Papavassiliou & Kaklamani* (4) found 89 7 per cent to retain their phage pattern after storage for 6 months, they kept their bacteria on slants at 4° C without subculture, and also without mentioning whether all strains survived such a prolonged period According to the present author's experience, it is likely that some strains died under such storage conditions for as long as 6 months Whether strains that tend to die out more easily have the most labile phage sensitivity patterns is not known

Our findings and those of others indicate that it is difficult to formulate rules for what variation in phage sensitivity pattern may be consistent with identity of pseudomonas strains In conformity with the convention in staphylococcal phage typing, variation in one reaction of + or ++ strength must be tolerated It is notable that 16 out of our 81 sequential cultures (19 7 per cent) of the same serogroup differed in two or more reac tions, but that the mean number of differen ces per set (1 2) still was considerably out-

numbered by the common reactions (3 3) *Sutter et al* (6) also studied sequential cultures from the same sites of patients and in 27 5 per cent observed ■ "major" variation in phage pattern

A difference in two reactions may be tolerated if similar origin is strongly suggested by other circumstantial evidence (serogrouping pyocine typing, and epidemiology) Certainly, variation in phages showing the least stable reactions would be less decisive Indeed, to verify affinity between bacterial isolates, it may be necessary to supplement phage typing with yet another epidemiological typing procedure

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BACTERIOPHAGE TYPING AND SEROGROUPING OF *PSEUDOMONAS AERUGINOSA* FROM ANIMALS

Tom Bergan

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Bacteriophage typing and serogrouping were carried out on 349 strains of *Pseudomonas aeruginosa* mainly from domestic animals A new bacteriophage set for *P aeruginosa* – which had been developed entirely on the basis of human strains – also worked satisfactorily in animal strains The percentage of non-typable strains isolated from animals was 7 9 as compared to 4 5 in a previous examination on 486 human strains Part of this investigation was carried out on isolates with known herd relationships, such that reservoir and transmission could be evaluated In several instances, humans had infections with pseudomonas of the same phage typing pattern and serogroup characteristics as simultaneously caused infections in cattle The ubiquitous nature of *P aeruginosa* as a pathogen was shown This induces interesting hypotheses regarding the zoonotic aspects of pseudomonas infections No particular phage types were characteristic for any particular animal species, or type, or site of infection—a finding which relates well with the omnipresence of pseudomonas The serogroup 0 6 was the most frequent among bovine and musteline strains, but this is in accordance also with the high frequency thereof in strains of human origin The most frequent phage typing pattern was 8, 12, 16 and related patterns (8, 8, 12, 12, 16, 8, 16 12 16) which together was found in approximately ⅓ of the isolates The significance of milking equipment as a reservoir and a vector of infection was indicated by the occurrence of the same bacterial types from equipment and from udders

Pseudomonas aeruginosa is a relatively rare cause of disease in animals The frequency, however, appears to be on the rise, particularly in mink (16) where pseudomonas infections may be rapidly spreading and fatal (5), and in bovine mastitis (15) which is contagious in some herds and at times has an unfavourable prognosis (22) During the 10-year period from 1953–1962, the number of mastitis specimens which yielded *P aeruginosa* rose from 1 per cent to slightly above 8 per cent of all cultures examined (14)

Epizootological aspects of *P. aeruginosa* in bovine mastitis have previously been investigated by serogrouping (19, 20, 25) which

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showed considerable sero homogeneity for the strains within a herd

Although no bacteriophage typing system has yet been developed particularly for such animal strains, phage typing also is a reasonable approach to characterize pseudomonas strains of animal origin

The purposes of this investigation were (a) to investigate how a new pseudomonas phage typing set particularly developed for human strains would perform on strains from various animal sources, and (b) to examine whether phage typing implemented any further subdivision of the serogroups of strains from herds of cattle Simultaneously, (c) the possibilities of a predominance for certain types in different foci of infection, or for particular host species were scrutinized

MATERIAL AND METHODS

Strains

Subjected to study were 349 strains of *P aeruginosa* obtained partly (i) from the Culture Collection at the Veterinary College of Norway (designated as VCC strains) where the strains originated from a variety of sources examined during the last 17 years and partly (ii) from the author's collection The latter refers to 17 musteline strains received in connection with a phage typing and serogrouping service extended to the Veterinary Institute of Norway There were 256 strains from cattle (mostly udder infections), milk, and dairying equipment When two or more strains from the same mammary quarter were included, the isolates were taken more than 14 days apart, occasionally after antibiotic treatment Further, there were 31 strains from other animals (chicken dog mink pig sheep), 49 strains from humans and 11 from water specimens

*Serogrouping**

The strains were serogrouped by a slide agglutination procedure (19 20) The twelve O serogroups of *Habs* (6) have been used for reference in this work The *Sandvik* group O II not represented within these groups has received the number O 13 in conformity with the notation of *Wahba* (26) and *Mikkelsen* (13) The sera employed were produced by the *Sandvik* antigens and procedure (19, 20) except for the groups O 2 and O 5 where the *Habs*'s strains (6) were used The O 14 serum was made with *Wahba's* original strain (26)

*) In the literature both the words *serotype* and *serogroup* are employed in connection with pseudomonas O-antigens This paper will in conformity to the salmonella terminology apply the term *serogroup* to the O-antigens, reserving the term *serotype* for flagellar antigens

Phage Typing

The phage procedures were as previously described (1) The typing set consisted of the phages 73, F7, M6, Me13, 113, F116, Px3, C1c, C4 C13 C21, H249, P10, VII, Z2, Z3 Z19, and Z20 as the primary set and the phages 21B, 68, Col 11 K9 and C15 as an auxiliary set Below these strains for convenience have been given the numbers 1–19 and Aux–1 to Aux 5 in the order of the above succession

RESULTS

Identity of Serogroups

On the basis of bovine strains *Thörne and Kyrkjebo* (25) extended *Sandvik's* (19, 20) system with a serogroup O VIII During the present investigation, this was shown to be identical to *Habs*'s serogroup O 8 as verified by absorption of O 8 serum with the O VIII type strain, this eliminated agglutination of the Habs O 8 type strain

Frequency of Serogroups

Table 1 shows amongst others that the serogroups O 3 and O 6 were the predominant ones among the animal strains, the latter accounts for ⅓ of the bovine herd strains Serogroup O 13 occured in ¼ of the VCC cattle strains and in 6 per cent of the VCC strains isolated from humans who had a close contact with cattle (cow tenders) Twenty one of the strains in the group "other animals" were isolated from mink Fifteen of these belonged to O 6 Several were possibly duplicate strains since they came from the same herd As evidenced by available information on the origin of the strains one could assume that 12 isolates were unrelated, of these, 8 belonged to serogroup O 6

To study the possibility of any trend in the relative frequency of the serogroups of animal strains, the results of grouping human strains from two other collections (1, 10) have been included in the Table 1

TABLE 1 *Distribution of Serogroups in 349 Strains of Pseudomonas aeruginosa of the Veterinary College Collection Compared to Other Studies of Mainly Human Strains (1 10)*

Source of bacterial strains	No of strains	Not tested	Self agglutinating	Not serogroupable	Serogroups 1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cattle	202	33	5	0	8	0	39	3	0	63	0	12	6	[illegible]	2	0	31	0
Milk	12	0	0	0	3	0	3	0	0	1	0	0	1	0	0	0	4	0
Milking equipment	42	4	0	0	3	0	11	1	0	6	0	0	0	[illegible]	0	0	17	0
Sum bovine herd strains	256	37	5	0	14	0	53	4	0	70	0	12	7	0	2	0	52	0
Per cent, bovine herd strains			2.5		6.4		24.2	1.9		32.0		5.5	3.2		0.8		23.7	
Other animals	32	8	1	0	2	1	0	0	2	15	0	0	1	1	1	0	0	0
Humans	49	16	1	0	2	0	9	1	0	15	0	0	1	0	1	0	3	0
Water specimens	12	0	0	1	0	0	3	0	0	7	0	0	0	0	0	0	1	0
Sum, non bovine strains	93	24	2	1	4	1	12	1	2	37	0	0	2	1	2	0	4	0
Per cent, non bovine strains			2.8	1.4	5.6	1.4	16.7	1.4	2.8	35.6			2.8	1.4	2.8		5.6	
Total	349	61	7	1	18	1	65	5	2	107	0	12	9	1	4	0	56	0
Per cent			2.4	0.3	6.2	0.3	22.3	1.7	0.7	36.8		4.1	3.0	0.3	1.4		19.2	
Mainly human strains of earlier study (1) per cent	486	0	7.4	6.2	5.6	6.2	18.1	1.4	10.3	17.5	0.8	2.5	6.2	2.9	3.3	0.4	1.0	5
Polish hospital strains (10), per cent	302	0	13.6	15.2	1.7	23.5	3.3	1.0	18.9	10.9	1.0	1.7	2.6	1.7	2.0	0	0.7	1

showed considerable sero homogeneity for the strains within a herd

Although no bacteriophage typing system has yet been developed particularly for such animal strains, phage typing also is a reasonable approach to characterize pseudomonas strains of animal origin

The purposes of this investigation were (a) to investigate how a new pseudomonas phage typing set particularly developed for human strains would perform on strains from various animal sources, and (b) to examine whether phage typing implemented any further subdivision of the serogroups of strains from herds of cattle Simultaneously, (c) the possibilities of a predominance for certain types in different foci of infection, or for particular host species were scrutinized

MATERIAL AND METHODS

Strains

Subjected to study were 349 strains of *P aeruginosa* obtained partly (i) from the Culture Collection at the Veterinary College of Norway (designated as VCC strains) where the strains originated from a variety of sources examined during the last 17 years and partly (ii) from the author's collection The latter refers to 17 musteline strains received in connection with a phage typing and serogrouping service extended to the Veterinary Institute of Norway There were 256 strains from cattle (mostly udder infections) milk, and dairying equipment When two or more strains from the same mammary quarter were included, the isolates were taken more than 14 days apart, occasionally after antibiotic treatment Further, there were 31 strains from other animals (chicken dog mink, pig sheep) 49 strains from humans and 11 from water specimens

Serogrouping*

The strains were serogrouped by a slide agglutination procedure (19 20) The twelve O serogroups of *Habs* (6) have been used for reference in this work The *Sandvik* group O II not represented within these groups has received the number O 13 in conformity with the notation of *Wahba* (26) and *Mikkelsen* (13) The sera employed were produced by the *Sandvik* antigens and procedure (19, 20) except for the groups O 2 and O 5 where the *Habs's* strains (6) were used The O 14 serum was made with *Wahba's* original strain (26)

Phage Typing

The phage procedures were as previously described (1) The typing set consisted of the phages 73 F7, M6, Me13, 113, F116, Px3, C1c, C4 C13, C21, H249, P10, Wl1, Z2, Z3, Z19 and Z20 as the primary set and the phages 21B 68, Col 11, K3 and C15 as an auxiliary set. Below, these strains for convenience have been given the numbers 1–19 and Aux 1 to Aux 5 in the order of the above succession

RESULTS

Identity of Serogroups

On the basis of bovine strains *Thörne and Kyrkjebo* (25) extended *Sandvik's* (19, 20) system with a serogroup O VIII During the present investigation this was shown to be identical to *Habs*'s serogroup O 8 as verified by absorption of O 8 serum with the O VIII type strain, this eliminated agglutination of the Habs O 8 type strain

Frequency of Serogroups

Table 1 shows, amongst others that the serogroups O 3 and O 6 were the predominant ones among the animal strains the latter accounts for ⅓ of the bovine herd strains Serogroup O 13 occured in ¼ of the VCC cattle strains and in 6 per cent of the VCC strains isolated from humans who had a close contact with cattle (cow tenders) Twenty one of the strains in the group 'other animals' were isolated from mink Fifteen of these belonged to O 6 Several were possibly duplicate strains since they came from the same herd As evidenced by available information on the origin of the strains one could assume that 12 isolates were unrelated, of these 8 belonged to serogroup O 6

To study the possibility of any trend in the relative frequency of the serogroups of animal strains, the results of grouping human strains from two other collections (1, 10) have been included in the Table 1

*) In the literature both the words *serotype* and *serogroup* are employed in connection with pseudomonas O antigens This paper will in conformity to the salmonella terminology apply the term *serogroup* to the O antigens, reserving the term *serotype* for flagellar antigens

the Veterinary College Collection Compared to Human Strains of Other Studies (1, 10)

Bacteriophages															
10	11	12	13	14	15	16	17	18	19	Aux 1	Aux 2	Aux 3	Aux 4	Aux 5	NT**
5	12	62	3	21	16	38	0	12	2	98	81	106	18	28	14
0	1	6	0	1	2	5	0	0	0	8	7	7	0	1	1
2	6	15	0	5	5	15	1	2	1	29	13	22	1	5	5
7	19	83	3	27	23	58	1	14	3	135	101	135	19	31	20
2 7	7 4	32 4	1 2	10 5	10	22 7	0 4	5 5	1 2	52 7	39 5	52 7	7 4	13 3	7 8
1	1	10	0	4	2	1	0	0	1	11	18	11	4	4	3
3	3	13	1	2	2	9	0	1	2	25	21	18	2	7	4
1	1	7	0	0	3	1	0	1	0	6	6	5	0	4	0
5	5	30	1	6	7	11	0	2	3	42	45	34	6	15	7
5 3	5 3	31 6	1 1	6 4	7 4	11 6	0	2 1	3 2	44 2	47 4	35 8	6 4	15 7	7 4
12	24	113	4	33	30	69	1	16	6	177	146	169	25	49	27
3 4	6 8	32 2	1 2	9 4	8 5	19 7	0 3	4 6	1 7	50 4	41 1	48 1	7 1	14 0	7 7
6	25	21	16	7	14	22	3	9	5	44	36	26	23	19	4
4 3	5 0	37 4	0 7	2 3	10 0	2 0	1 7	3 3	7 9	10 3	0 7	6 3	1 0	4 3	16 6

** Non typable

typed Two isolates with serogroup 3 and phage pattern 2, 11 (herd C) were found in chloramine washing used to decontaminate the milking machines The same types as in the humans were found in specimens from equipment and also from udder and milk

It may be added that in the whole material *i e* including also isolates not included in Table 4, the three single most frequent phage sensitivity patterns were 8, 8 12, 16, and 8 12 found *in 43 24*, and 24 isolates each Phage sensitivity patterns related to these, 12, 16, 8, 16, 12, and 16, occurred in an additional 26 isolates such that this complex of sensitivity patterns was found in a total of 117 cases These strains were found both in humans, cattle, mink pig sheep and in milking machine utensils Thus, they are not to be considered as typical of the dairy environment

As a by product of this investigation, the variation noted in the bovine herd strains is of interest Table 4 suggests that a variation in two strong reactions (+ or + + reactions (1)) may be compatible with the same original source The results of phage typing for strains that are known to represent repeated subcultures from the same cow are shown in Table 5

DISCUSSION

The most remarkable finding was the fact that the new pseudomonas phage typing set, which had been developed primarily for human strains (1), gave a high percentage of typable strains (92 per cent) also among animal strains

Experience has shown a varied phage susceptibility for staphylococci from different animal species Thus, with the international 'human' phage typing set at routine test dilution (RTD), 24 per cent of 618 canine cultures (3) and 7 per cent of bovine strains (27) were typable *Watson* (27) found 7 9 per cent of 278 bovine *Staphylococcus aureus*

TABLE 3 *Frequency Distribution of Concordance between Serogroups and Phage Types for 348* Pseudomonas aeruginosa *Strains of Primarily Animal Origin*

	Serogroups															
Phages	1	2	3	4	5	6	7	8	9	10	11	12	13	14	SA*	Sums
1			1			19									2	22
2	2		26			15									3	46
3			4			6									1	11
4	7		3			3										13
5	1		13			1									1	16
6		1	5			10							1		1	18
7	1		22			9			1		1				5	38
8	8		23		1	72		2	7		1		37		2	153
9	2		13			1										16
10	1		1			3					1		2			8
11			15			2		2					2			21
12	13	1	15		2	25		1	7		1		33		2	100
13						2			1							3
14	1	1	15			8		1	1							27
15	[illegible]		11			6							1		1	25
16	6		12			10							27			55
17													1			1
18	1		4			4		1	2		1					13
19		1	1			3										5
Aux– 1	9		56			31		4	3		2		32		4	141
Aux– 2	12		29	1		60		1	4		1		1		2	111
Aux 3	11		56			36		3	6				15		3	130
Aux 4			5			13										18
Aux– 5			19			8		1	6				1		1	36
NT**				4		2		5					10		1	2[illegible]
Sums	81	4	349	5	3	349	0	21	38	0	[illegible]	[illegible]	163	0	29	

* SA = self agglutinable
** NT = non typable
The table shows the number of bacterial strains lysed by each phage and the number of serogroups to which these bacteria belonged The figure zero has been omitted in the empty positions

strains to be typed by the human phage set at RTD *Davidson* (4) with the human typing set lysed 65 per cent of bovine strains but a higher percentage among human strains With as many as 43 "bovine" phages (*i e* phages from lysogenic strains of bovine origin), *Davidson* at RTD found 34 per cent among human and 91 per cent of bovine staphylococci to be typable *Live* & *Nichols* (11) found 4 9 per cent among canine staphylococci to be typable by the human set *Jaekel et al* (9) after literature studies concluded that approximately 25 per cent of canine staphylococci could be typed by the international human set With 5 canine phages at RTD, they (9) succeeded in typing 55 per cent among dog staphylococci whereas only 12 per cent were typable among human staphylococci and 18 per cent among bovine strains This shows that for staphylococci it is necessary to develop separate phage typing systems for strains of different animal origin

Ecological differences for staphylococci have been substantiated by biochemical and biophysical tests on strains of human bovine leporine and poultry origin *(18)* *Indeed* differences in virulence have been demonstrated in that antibiotic resistant staphylococci of a certain phage type *pattern* elicited an endemic of furuncles, cellulitis, abscesses and paronychias in veterinary students who handled asymptomatic animals which in the anterior nares harboured bacteria of the same type (17)

TABLE 4 *Differentiation by Phage and Serogrouping of Pseudomonas aeruginosa in Some Herds of Cattle*

Herd	Total no of strains	Serogroup (no)	Phage pattern ‖	Source (no)
A	11	5(11)	1, 2, 6, 9, 14, 18, 19	Udder (1)
			3, 7, 8, 9, 10, 14, 15, 16, 3, 4, 7, 8, 9, 14, 15, 16, 4, 7, 8, 9, 12, 14, 15, 16, 7, 8, 9, 14, 15, 16, 7, 8, 9, 14, 15, 7, 8, 14, 15, 16, 8, 14, 15, 16	Udder (5), Equipment (4)*
			6, 7, 14, 15, 16	Udder (1)
B	6	6(6)	1, 8, 7, 8, 8	Udder (3)
			2, 3, 6, 8, 10, 13, 15, 2, 3, 8, 10, 15	Udder (2)
			Aux-2	Udder (1)
C	85	ND(11)§	8, 12, 16	Udder (1)
			16	Udder (8), Human (2)
		1(9)	4, 12, 4, 12, 16, 4, 12, 15, 16, 12, 12, 15, 16	Udder (2), Milk (3), Equipment (3), Human (1)
		3(21)	2	Human (2), Udder (1), Equipment (1)
			2, 11, 2, 5, 11, 2, 3, 11, 18, 2, 5, 15, 2, 5, 8/15, 2, 5, 7, 8, 12	Udder (10), Milk (2), Equipment (2), Human (2), Chloramine wash (2)**
			8, 12, 16	Udder (1), Equipment (1), Human (2)
			7	Human (1)
			12	Equipment (1)
			Aux-1, 3	Udder (1), Equipment (1), Human (1)
		8(1)	Aux-1	Nose, calf (1)
		13(37)	8, 8, 12 8, 12, 16, 8, 16, 8, 10, 16, 8, 10, 8, 11, 12, 16 8, 12, 15, 16	Udder (20), Milk (2), Equipment (12), Human (3)
D	23	ND(2)	7, 8	Udder (2)
		6(21)	2, 6, 14	Udder (1)
			4, 8, 16, 8, 8, [illegible], 16, 18 8, 12 16, 8, 16	Udder (17), Equipment (1)
			14	Udder (1), Equipment (1)
E	15	ND(1)	NT‡	Udder (1)
		4(3)	NT	Udder (2), Equipment (1)
		13(11)	NT	Udder (3), Equipment (1), Milk (1)
			Aux-1, 3 Aux-3	Udder (6)
F	5	1(4)	8, 12	Udder (4)
		8(1)	NT	Udder (1)
G	5	ND(1)	8, 9, 12, 14	Udder (1)
		3(3)	5, 7, 8, 9, 12, 14, 18 8, 9, 12, 14	Udder (2)
			Aux-3	Udder (1)
		13(1)	12	Udder (1)

TABLE 4 *continued*

Herd	Total no of strains	Serogroup (no)	Phage pattern‖)	Source (no)
H	5	8(5)	8, 12, 14	Udder (1)
			11	Udder (1)
			NT	Udder (3)
I	5	ND(1)	5, 7, 8, 12	Udder (1)
		3(1)	2, 5	Udder (1)
		9(3)	8, 8, 12	Udder (3)
J	8	6(7)	1, 2, 7,	Udder (1)
			3, 5, 7, 12/8, 9	Udder (1)
			8 12	Udder (6)
K	14	ND(7)	2, 6, 8 2, 6 8 13, 8	Udder (7)£)
		4(1)	NT	Udder (1)
		13(6)	NT	Udder (2), Equipment (3)
			6	Equipment (1)
			12	Udder (1)

*) = *Milking machine equipment*
§)ND = Not serogrouped
**) = Chloramine disinfectant used for washing *milking machine and tubings* The phage pattern of these two strains was 2 11 which is the same as was found in six samples from udders and two samples from equipment
$)NT = *non typable by the bacteriophage set*
£) – All specimens from same cow
‖) – Phage sensitivity patterns which by virtue of their similarity are sufficiently overlapping to indicate that the isolates are probably progeny of the same parental strain have been grouped together Different patterns within these groups are separated by semicolon For each such group of patterns in the column to the far right is given the sum of the types and number of sources for all isolates with any of the phage sensitivity patterns concerned

Differences in susceptibility to 'human" and "animal phages have also been noted for *E coli Smith & Crabb* (24) found between 77 and 93 per cent of *E coli* strains from cattle to be typable by a typing set developed for bovine strains, correspondingly, the percentage of typable strains from humans was 34, from pigs 46 from sheep 12, and from poultry 70

When as much as 92 per cent of the VCC strains were typed, this is of considerable importance Strains of veterinary origin had purposely been excluded from the bacteria on which the new pseudomonas typing set was developed since it was constructed particularly with human epidemiology in mind The current findings may reflect the ubiquitous nature of *P aeruginosa* Any tendency towards the development of an ecological niche for pseudomonas appears to be minimal Pseudomonas strains seem not to be specific for any particular animal species This could be a consequence of the primarily saprophytic nature of this microbe It is significant to note that identical pseudomonas sero groups and phage typing patterns could be isolated from active infectious processes both in cows and in humans (otitis) in the bovine herd environment (*Herd C, Table 4*) This emphasizes the zoonotic aspect of pseudomonas infections

In one herd (C), pseudomonas strains of the same characteristics were found in water of a stream used for watering the pastures where the cattle were grazing in the cattle and in the specimens from a herdsman (hand and nose) The water strain unfortunately was not available for phage typing but belonged to serogroup O 13 Water was considered a possible source of pseudomonas infections by *Sandvik* (20), *Thörne & Kyrkjebo* (25), and *Haagsma & Pereboom* (5), but in

TABLE 5 *Phage Typing Patterns Obtained on* Pseudomonas aeruginosa *Isolates from Different Specimens Each Taken at Least a Fortnight Apart from the Same Cow.*

Subject	Phage pattern	Number of isolates
Cow 1	8	2
	2 8	1
Cow 2	8 12	1
	6 8 9 14 18	1
Cow 3	8	4
	2 6 8	1
	2 6 8 13 19	1

this instance the bacteria could also have been passed *from the animals to the water* An interesting observation (Table 4) was that although a single (or a few) serogroup(s) tended to predominate in a herd phage typing rendered a further subdivision into subsets of related strains and thus would allow a monitoring of the spreading of an endemic

In several instances (Table 4) there was identity in serogroup and also overlapping phage sensitivity patterns of pseudomonas strains isolated both from (a) udders and from (b) milking machines milking utensils, and milk Simultaneous occurrence of pseudomonas in milking equipment and udders has been noted previously (7 20 22 25) *Sandstk & Skulberg* (21) observed pseudomonas *in the milking* machines of 3 among 25 herds Another important source of infection was seen in herd D where pseudomonas was isolated from chloramine solution used for rinsing the equipment The role of chlorine disinfectants in the prevalence of pseudomonas infections has been made obvious previously by field sampling (21) and is related to the inferior disinfecting power of chloramine and hypochlorites as has been amply documented (2)

Bovine mastitis is a herd problem A summary of relevant literature has been presented by *Nilsson & Thorne* (15) The transmission paths between udders, milking equipment milk chlorine disinfectants and humans could be suspected also *on the basis of* *Sandstk's* (20) serogrouping of bovine herd strains

Although alternative paths of transmission may be outlined, the route of the initial introduction of pseudomonas in a herd is currently subject only to speculation The present *findings of strains with* the same sero- and phage characteristics in human herd tenders as in herds do not elucidate the direction of spread between man and animal With a lack of rigid barriers regarding susceptibility to the same pseudomonas types between different animal species it could be suspected that pseudomonas was introduced in *e g* a bovine herd by an enteric human carrier *Shooter et al* (23) found 24 per cent among 249 humans admitted to a surgical department to be faecal pseudomonas carriers Humans as a primary source have been implicated by *Hoadley & McCoy* (8) who stated that pseudomonas was found mainly in animals associated with man *Hoadley & McCoy* found 13 per cent human and 25 per cent canine faecal carriers Faecal carriers have also been noted among calves, but not among mature cows (8 12) Thus, there is ample possibility that in the material at hand, the pseudomonas strains may have spread from man to beast but the other direction of spread is also possible

The frequency of bacterial strains lysed by each phage was recorded for all strains typed (Table 2) The significance of the circumstance that the phages 2 6 8 and 12 were distinctly more lytic for animal derived strains than on strains derived from humans is difficult to understand For phage 19 the situation was reversed Table 2 shows the remarkable discrepancy in the lytic activity for the phages 13 16 and the auxiliary phages on the two collections (1 10) of mainly human strains

The serogrouping rendered the interesting result that group O 13 was mainly derived from animals and that 0 6 accounting for ⅓ of all isolates was the most frequent single group in the bovine strains Serogroup O 6 and the cross reacting group O 14 contributed 23 per cent of the 486 human strains of one collection (1) and 12 per cent of another consisting of Polish hospital strains (10)

(Table 1) Serogroups O 2 and O 5, which were not found at all in the cattle and in but 1 of the 21 musteline strains, accounted for 6 and 10 per cent respectively in the 486 human strains (1), 24 and 19 per cent in the Polish strains (10) Notable is also that there were more untypable and self agglutinable strains in the human materials

The variation in lytic activity found and the distribution in serogroups in the VCC strains as compared to the strains of the two human collections thus indicate that any differences between the strains of animal origin and those of human derivation are probably chiefly the result of a local epidemiological predominance of particular bacterial strains and perhaps not indicative of fundamental host preference for certain types of *P aeruginosa*

Wokatsch (28) extended the serogroup system of Habs by 13 additional groups In his experience on strains of mainly bovine origin, the most frequent groups were O 3, O 1, his O 13, O 2/5, and his O 14 He found 7 per cent belonging to serogroups O 2/5, the figure was raised to 12 6 per cent if his groups O 15 and O 25 which cross reacted with O 2/5, were included It is interesting to note that serogroup O 6 was the most frequent entity (i) in *Habs*s (6) material ($^1/_6$ of the strains) (ii) in the 486 human strains (¼) (iii) in the VCC strains (O 6 accounted for ⅓ of the strains), and (iv) in *Sandvik*s (19 20) material from bovine mastitis This contrasts with *Wokatsch* (28) who noted this entity in only 3 5 per cent of the strains derived from animals *Wokatsch*s remarkable lack of the serogroups 11 and 12 was almost reproduced in the VCC strains among which only 3 animal and 1 human strain belonged to O 11 In reference (1) it constituted 3 7 per cent of the strains It is nevertheless, difficult to promulgate any particular serogroup or phage type as typical of any animal species or type (or site) of infection

Only in mink was there a tendency towards one predominantly more frequent serogroup O 6 In this connection the finding that O 6 was also the most frequent in man is to be noted but to what extent animal infection actually comes from man remains to be documented Anyhow, the high frequency of O 6 as a cause of the rapidly spreading and predominantly fatal pseudomonas infections in mink has therapeutic significance since it makes immunization a rational approach for protection of entire musteline herds This has been tried with apparant success by Dutch workers (5)

In bovine herds and in mink farms where pseudomonas infections have been a problem a *routine surveillance by serogrouping* and phage typing of pseudomonas isolates is to be recommended This allows a tracing of a spreading infection and may aid in the search for pathogenic strains If the hitherto observed serological homogeneity of pseudomonas from mink continues typing also has a direct bearing on attempts to vaccinate herds of mink

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HEPATITIS-ASSOCIATED ANTIGEN IN PREGNANT WOMEN

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Hepatitis associated Antigen (HAA) was detected in 82 out of 69,978 Danish pregnant women studied (0 12 per cent) This incidence varied from 0 36 per cent in some metropolitan areas to 0 03 per cent in rural districts Only one of the antigenaemic women had acute hepatitis, 81 were apparently healthy carriers The pregnancies did not imply any deterioration of the healthy carrier state HAA could not be demonstrated in any of 51 cord blood samples studied and the children remained antigen negative and healthy during a 4–12 month follow up

Viral hepatitis during pregnancy is an uncommon complication which might be associated with an increased incidence of abortion and premature delivery (8, 17) Transmission of the disease to the offspring is still under discussion (1, 8, 21) as neonatal hepatitis is assumed to be a multi aetiologic syndrome (5) Some such cases, however, are obviously caused by hepatitis virus (1, 21)

Determination of hepatitis-associated antigen (HAA) which [illegible] intimately related to long incubation hepatitis virus (11, 18) now provides a possibility to study the transmission of this disease from pregnant women to their babies

A few larger investigations, however have shown somewhat contradictory results indicating that HAA does not usually cross the placental barrier (15, 20), but on the other hand, it has been shown to occur in 7 per cent neonates with various liver disorders (1

The present investigation [illegible] a prospec study of pregnant women in whom HAA detected early in pregnancy

MATERIAL AND METHODS

By law, all Danish pregnant women have a bl sample taken as soon as their pregnancy [illegible] verti to be tested for the Wasserman reaction Dur a ten month period, sera from pregnant wome collected from the whole country, were investigat for HAA The test was performed by gel-electr phoresis as previously described (19) The an ... antise ... Nation

... tenti

by geldiffusion using the reference sera HA positive sera were identified and the physicians r quested to perform a clinical investigation and obtain further blood samples at the patients r gular visits and at delivery

The sera were investigated for HAA alani aminotransferase (GPT) bilirubin, alkaline pho phatases and serum electrophoresis including quan titative determination of IgG, IgA and IgM, b the standard methods of the laboratory

Cord blood from the children was also examine

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genetically The results of this study will be lished separately The children were, as far as sible investigated at monthly intervals for cal signs of liver disease and for HAA anti A and GPT Most children were followed for months and some were re investigated at one r of age

RESULTS

ring a ten month period, 69,978 sera were estigated and a positive reaction for HAA s obtained in 82 women (0 12 per cent)

Country of origin 75 of the women were rn in Denmark, four were from the Medi ranian area one from Latin America, one om another Scandinavian country and in le case the origin could not be determined

Place of residence An unequal distribution the HAA positive women was found as ost of the women lived in the metropolitan rea The frequency of HAA positive women different areas is given in Table 1 One oman could not be traced

ABLE I *Geografical Distribution of HAA Positive Pregnant Women*

Area	HAA positive no	%
Rural Districts	9	0 03
Provincial Towns	30	0 15
Metropolitan Area	42	0 21
(Densely populated Districts of Copenhagen > 150 inhabitants/hectare)	(16)	0 36
Whole Denmark	82	0 12

As seen the frequency in the Copenhagen area was seven times greater than in the rural districts and within the three most densely populated districts (more than 150 inhabitants/ha) the frequency was 12 times greater than in the rural districts

Age The HAA positive women were all between 17 and 33 years old which is significantly lower than the expected age distribution for child bearing mothers in Denmark ($p = 0\,0014$)

24*

If seven women working in hospitals are omitted from the material the deviation is no longer significant

Marital status and occupation The marital status did not differ from the total population group concerned Regarding occupation, it was notable that seven (8 7 per cent) of the HAA positive women were hospital employees

Acquisition of HAA From the case histories of the HAA positive women, detailed stories regarding hepatitis were obtained from 59 Among these, only two had a known history of jaundice Possible exposure to HAA included four cases who had received blood transfusion, eight cases with jaundice in the family and three cases who lived with a drug addict None of the women were drug addicts themselves

Hepatitis during pregnancy Only one woman had signs of hepatitis when HAA was detected She was admitted to a hospital where repeated liver biopsies confirmed the diagnosis All other women in the study had normal aminotransferase values during their pregnancy In connection with the delivery however, one woman had a short asymptomatic increase in the GPT value, but liver biopsy was not performed In most of the women a physiological increase of the alkaline phosphatases was observed during the last month of pregnancy In eight women a persistent increase of IgG was found but none of these showed any other signs of chronic liver disease

Three of the apparently healthy women underwent liver biopsy in another hospital One showed mild hepatitis and two slight unspecific inflammation

None of the women suffered from any other disease

Regarding the HAA reaction, the majority of the women had persistent positive reactions throughout the investigation Only in six cases did the reaction become negative during the pregnancy In two women however only one serum sample was tested

The outcome of the pregnancies Out of the 82 women two were lost to the investiga

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Furthermore, all positive reactions were verifie by geldiffusion using the reference sera HA positive sera were identified and the physicians re quested to perform a clinical investigation and t obtain further blood samples at the patients re gular visits and at delivery

The sera were investigated for HAA, alanine aminotransferase (GPT), bilirubin, alkaline phos phatases and serum electrophoresis including quan titative determination of IgG IgA and IgM b the standard methods of the laboratory

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titis at the time of delivery and those born to HAA-carriers

In the first group, a total of eight cases has been investigated and in all these the children developed antigenaemia (4 16, 19, 23, 25) In two of the children it was followed by signs of hepatitis and in one of these it progressed to cirrhosis of the liver (25) In the carrier group, seven cases have been published previously, but only one child became HAA positive (4, 6, 13, 20)

All children studied in the present investigation were born to HAA carriers and all remained HAA negative during the 4 to 12 months' follow up period Hence it might be concluded that HAA from healthy carriers is only very rarely transmitted to the newborn child The reason for this apparent failure of transmission might be that the children are immunized against HAA during pregnancy, but so far, anti HAA has not been detected either in cord blood or in serum samples obtained in the follow up period From the present investigation it appears safe to conclude that the presence of HAA carriership in pregnant women does not imply any increased risk for the mother or for the child Hence special precautions do not seem to be indicated in these cases

We are greatly indepted to Mrs *I Frandsen* who performed the numerous antigen tests

Also we wish to thank all the patients' physicians, the Departments of Pediatrics in *Arhus, Odense* and Esbjerg and the Blood Bank at the Copenhagen Serum Institute for their help and supply of sera. The study was supported by a generous grant from *The Research Committee of the Danish Mental Retardation Service*

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THE INFLUENCE OF PREPARATION TECHNIQUE, HUMIDITY AND IRRADIATION CONDITIONS ON RADIATION INACTIVATION OF *STREPTOCOCCOS FAECIUM*, STRAIN A_21

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Cobalt-60 gamma radiation has a greater inactivating effect on dried preparations of *Str faecium*, strain A_21, than electron radiation from a 10-MeV linear accelerator The difference in effect varies with relative humidity and preparation technique The difference is 10–20 per cent when the bacteria are dried with serum broth at 10–50 per cent r h The radiation resistance under these conditions corresponds to the resistance of standard preparations of this strain for control of microbiological efficiency of radiation sterilization plants Increase of the relative humidity to 55 per cent leads to a significant drop in radiation resistance, and a doubling of the difference in effect between gamma irradiation and electron irradiation The response of cleaned preparations is less dependent on relative humidity and very similar to the response observed at high humidity in preparations with serum broth No difference in the effect of the two irradiation sources is observed when the bacteria are irradiated in aqueous suspension or suspended in serum broth

Radiation sterilization of medical equipment is mainly carried out in gamma irradiation plants or with electron accelerators

In 1966 *Christensen* observed (3) that cobalt-60 gamma irradiation had a greater inactivation effect on dry test pieces with *Streptococcus faecium* strain A_21, than irradiation with 10 MeV electrons from a linear accelerator Test pieces with this strain are used as one of the reference standards for control of microbiological efficiency of radiation sterilization plants according to the "IAEA Recommended Code of Practice for Radiosterilization of Medical Products" (10)

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In the Scandinavian countries, the choice of dose for radiation sterilization has mainly been based upon the radiation resistance of *Str faecium* (4, 5) because it is the most radiation-resistant potentially pathogenic strain so far isolated from medical equipment or factory environments A practical consequence of the dose rate effect in this strain has been that cobalt-60 gamma irradiation plants used in Scandinavia for radiation sterilization apply doses 10 per cent lower than those used with electron accelerators

Christensen (2) has previously found that the radiation resistance of two strains of *Str faecium* and of two strains of *Bacillus subtilis* varied much when dried from different suspension media and after different predrying manipulations The highest radiation resistance in these investigations was found when the micro organisms were dried uncleaned and protected by organic substances It is well known that also changes in water content and availability of oxygen can cause changes in radiation resistance of dried bacteria (13, 19)

It is, however, not known how much such factors contribute to the observed difference in effect between gamma irradiation and accelerator irradiation on *Str faecium*, strain A-1, and how much difference in effect may vary with variations in preparation technique Such knowledge is essential for a safe evaluation of the relative microbiological efficiency of different radiation sterilization plants

MATERIALS AND METHODS

Preparation of Test Samples With a few modifications culture preparation and plate count techniques were the same as those applied with test pieces for efficiency control (2)

Streptococcus faecium strain A 1 (ATCC No 19581) was cultured on 5 per cent blood agar at 30–32° C for four days The cultures were then scraped off the plates and suspended in serum broth 0.02 ml droplets of the broth (holding around 10^{8} viable units) were placed in small 1 ml glass cells *In some experiments the bacteria were cleaned by means of five centrifugations in 0.9 per cent saline and then suspended in distilled water* Droplets of the water suspension were then placed in glass cells as above The glass cells were immediately predried in the air stream of a laminar air flow cabinet for two–four hours (weak predrying)

The glass cells were then held in equilibrium at 25° C overnight in a glove box with a saturated salt solution or a calibrated sulphuric acid corresponding to the desired relative humidity The glass cells were closed with an airtight stopper and removed from the box for irradiation Irradiations and platings were carried out within forty-eight hours In some experiments using cleaned bacteria the air in the glove box was exchanged for nitrogen

In some experiments the glass cells were held at 30 per cent r h overnight (hard predrying) and then equilibrated to a higher humidity level The experiments referred to as 0 per cent r h were carried out with glass cells evacuated to 2×10^{-3} mm Hg

Electron irradiations were performed with the bent beam of the 10-MeV linear accelerator at Risø The pulse frequency was 300 pulses/sec, and the pulse width 7 microseconds The dose in the pulse was 7 Krads The total absorbed dose was measured by calorimetry (15)

Cobalt-60 gamma irradiations were carried out in the cobalt-60 plant at Risø which has been calibrated with the Fricke dosimeter The dose rate varied from 0–2 Mrads/h as the samples were conveyed through the irradiation field (15)

All irradiations were carried out at a temperature of 25° C except for irradiations of bacteria in suspension which were carried out at 0° C in order to minimize metabolic activity The suspended bacteria were kept in hermetically closed, 10 cm long and 0.1 cm wide polyethylene tubes with no head space

The criterion for survival of the bacteria was the colony forming ability after 4 days on 5 per cent blood agar at 30–32° C At least three totally independent estimates of dose survival curves with a minimum of 8 glass cells were carried out at each dose rate and humidity level

The radiation inactivation curve for *Str faecium* has a sigmoidal shape and has neither a well defined shoulder nor a well defined final slope We used the radiation dose that reduces the surviving fraction to 10^{-4} (LD 99.99) as a convenient parameter for comparison of radiation resistance under the different experimental conditions

RESULTS

In all experiments with dried preparations of *Str faecium*, strain A-1, the inactivating effect of electron accelerator irradiation was lower than the effect of gamma irradiation Changes in irradiation conditions caused smaller variations in the radiation response in accelerator experiments than in experiments with gamma irradiation

The shape of inactivation curves for all accelerator irradiations of dried preparations were similar to that of curves for test pieces for control of microbiological efficiency of irradiation sterilization plants (Fig 1), where as the curves obtained with bacteria suspended in serum broth or water as well as with gamma irradiation of cleaned bacteria in dried preparation had somewhat different shapes These curves are also sigmoidal but

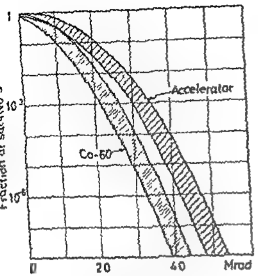

Fig 1 Bands of inactivation curves for electron accelerator irradiation and cobalt-60 gamma irradiation of *Str. faecium*, strain A_21, in the serum broth system at 10–50 per cent r.h.

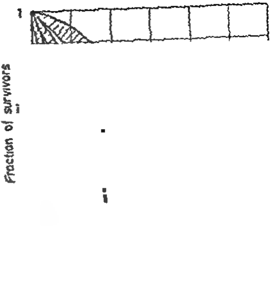

Fig 2 Bands of inactivation curves for electron accelerator irradiation and Cobalt-60 gamma irradiation of *Str. faecium*, strain A_21, in dry cleaned preparations and in aqueous suspension

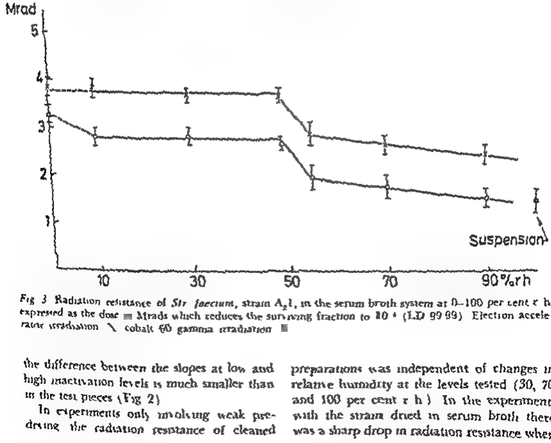

Fig 3 Radiation resistance of *Str. faecium*, strain A_21, in the serum broth system at 0–100 per cent r.h. expressed as the dose in Mrads which reduces the surviving fraction to 10^{-4} (LD 99.99). Electron accelerator irradiation \ cobalt-60 gamma irradiation ■

the difference between the slopes at low and high inactivation levels is much smaller than in the test pieces (Fig 2)

In experiments only involving weak predrying the radiation resistance of cleaned preparations was independent of changes in relative humidity at the levels tested (30, 70 and 100 per cent r.h.) In the experiments with the strain dried in serum broth there was a sharp drop in radiation resistance when

TABLE 1 *LD 99 99 in Mrads for Radiation Inactivation by Electron Accelerator Irradiation and Cobalt 60 Gamma Irradiation of* Str faecium, *strain A_2I, in Preparations with Serum Broth (SB) and in Cleaned Preparations (CL) at Different Humidity Levels and Preparation Techniques*

Preparation technique	Radiation source	
	Accelerator	Cobalt-60
0 per cent r h (SB)	3 7±0 3	3 2±0 3
10–50 per cent r h (SB)	3 7±0 2	2 8±0 2
55–100 per cent r h. (SB)	2 6±0 2	1 8±0 2
30–100 per cent r h (CL)	2 7±0 2	1 7±0 2
Nitrogen atmosphere (CL)	2 8±0 2	1 5±0 2
Hard predrying 100 per cent r h (CL)	3 0±0 6	0 7±0 1
Hard predrying 100 per cent r h (SB)	—	1 0±0 1
Suspension (SB)	1 1±0 2	1 2±0 3
Suspension (CL)	0 7±0 1	0 7±0 1

the humidity was increased from 50 per cent to 55 per cent r h (Fig 3) The inactivation curves at 10–50 per cent r h were identical to the inactivation curves for standard test pieces (Fig 1) The inactivation curves at 55–100 per cent r h were very similar to the inactivation curves for the cleaned preparations (Fig 2) A similar abrupt change in radiation resistance at a somewhat lower humidity level has been observed in studies using spores of *Bacillus megaterium* (13)

Irradiation of the bacteria in dry serum broth under vacuum gave approximately the same response as at 10–50 per cent r h, there was a slight increase in the resistance for gamma irradiation but there was still a clear difference in effect between gamma and accelerator irradiation (Table 1)

The radiation resistance of dried clean preparations with exclusion of oxygen was the same both for gamma and electron accelerator irradiations as in the experiments where the small compact preparations were surrounded by atmospheric air (Table 1)

When the bacteria were equilibrated to 100 per cent r h after hard predrying their *resistance to gamma* irradiation would be reduced by about fifty per cent whereas the resistance to electron accelerator irradiation remained almost unchanged (Table 1)

No difference in the effect of the two radiation sources could be observed whether the bacteria were irradiated suspended in water or serum broth (Table 1)

The protective effect of serum broth both in the experiments using suspended bacteria and in the experiments carried out at 50 per cent r h and below, is of the same magnitude as that reported for many strains of bacteria with broth and various specific compounds (2 11 14, 16 18, 19) The protective effect of serum broth was absent at 55 per cent r h and higher humidity levels Disappearance of protection at certain humidity levels has also been seen in experiments using spores of *B subtilis* var niger (16) (chelatin) and *Serratia marcescens* (18 (inositol), but the variations in protection as well as in radiation resistance followed patterns other than those in this experiment

DISCUSSION

It has not been very usual to apply vegetative bacteria for microbiological control of sterilization of medical supplies The microbiological control of the classical sterilization procedures is normally carried out by means of test preparations containing a great number of spores of a relevant spore forming strain The choice of test strain depends on the sterilization process *to be controlled* A relevant reference strain for a given sterilization procedure should be more resistant to the

process than all commonly occurring micro-organisms likely to be found in the relevant environment, and more resistant than all pathogenic micro-organisms (5)

Because vegetative bacteria may have higher radiation resistance than spores, the radiation inactivation of these micro organisms must also be considered in evaluations of the safety of radiation sterilization As previously mentioned *Str faecium*, strain A-1, fulfils the criteria as a reference strain for radiation sterilization

The basic demands to reference standard preparations for control of sterilization of medical equipment are

(1) Optimal resistance
(2) Reproducible resistance
(3) Storage stability

The results reported here show clearly that it is not sufficient simply to recommend *Str faecium*, strain A 1, as a reference strain for radiation sterilization of medical equipment The radiation resistance varies with preparation technique, and it is therefore necessary to refer to specific standard preparations produced by specified laboratories Besides, by the parameters investigated here, variations in the inactivation curves may be caused by factors such as growth conditions for the cultures used for test piece preparation and growth conditions at the evaluation of number of surviving organisms of irradiated test pieces (9) Variations in post irradiation effects and storage stability may also be expected with variations in preparation technique

In this investigation the highest radiation resistance of *Str faecium* strain A-1, was found in the serum broth system at humidity levels below 50 per cent r h both for gamma and accelerator irradiation The radiation resistance corresponded to that known from test pieces produced at ambient humidity Some of the batches which we produced at ambient humidity, however, had lower resistance and short storage life, but the data here show that this most probably was due to a too high humidity level in the laboratory at that time Test pieces for control of microbiological efficiency are now produced at 30 per cent r h

A production of test pieces with *Str faecium*, strain A-1, based on a technique involving the washing procedures used here for the cleaned preparations may improve batch to batch reproducibility at ambient humidity, and may therefore have some practical advantages Estimates on the sterilizing effect of radiation plants based on such test pieces, however would be far too optimistic The efficiency of electron accelerator irradiation would be overestimated by 10–20 per cent, and the efficiency of Cobalt 60 gamma irradiation by 30–50 per cent

The data presented here support that it may be justified to assume that Cobalt-60 gamma irradiation has a 10 per cent higher sterilization effect than electron accelerator irradiation when *Str faecium*, strain A-1, is applied as reference standard The results however, fail to give a clear indication of the reason for the difference in effect although it seems quite evident that water and protective substances separately or together, are important factors

The most apparent difference between a gamma sterilization process and an electron accelerator sterilization process which could cause a difference in inactivation effect is the dose rate Differences in inactivation effect on bacteria of radiations at different dose rates have been attributed to oxygen depletion (1, 6, 7, 8, 12, 17), but oxygen is apparently not involved in radiation inactivation at the experimental conditions in this work, probably because the doses applied were very much higher than those otherwise known to remove oxygen from water or tissue (40 krads–200 krads) (1) This does not rule out the possibility that the difference in inactivation effect may be related to the dose rate by some other mechanism as for example reduced damage to essential biomolecules at the high dose rate because of radical—radical reactions

Work with different types of electron ir

radiation is now in progress in order to see whether there is a relation between dose rate and inactivation effect

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THE SYNTHESIS OF PHOSPHOLIPASE C BY *BACILLUS CEREUS* AND ITS RELATION TO SPORULATION

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Some conditions for the synthesis of phospholipase C by *Bacillus cereus* have been investigated Nutrient broth dialysate, containing only substances of low molecular weight, was a convenient growth medium when starting material for the purification of phospholipase C was desirable An unidentified low molecular medium component completely inhibited the synthesis of phospholipase C Sporulation and growth rate were not detectably influenced by the presence or absence of the enzyme

Phospholipase C (EC 3 1 4 3) is an exo-enzyme produced by some members of the family *Bacillaceae* It has recently acquired importance as a tool in studies of membranes and membrane bound enzymes (2, 8) We report some studies on the synthesis of phospholipase C by *Bacillus cereus* (ATCC 10987) under various growth conditions Only low molecular, i e dialysable, medium components were necessary, greatly facilitating subsequent purification

MATERIALS AND METHODS

Phosphorylcholine phosphorylethanolamine and phosphorylserine were obtained from Sigma

The components of Brain Heart Infusion (Beef Heart Extract and Calf Brain Extract) were kindly provided by Oxoid Ltd

The media used were

1 Nutrient broth (N b) (Oxoid) 13 g/l
2 N b dialysate (Oxoid) 26 g/l
3 N b (Difco) 13 g/l
4 K_2HPO_4 7 g/l KH_2PO_4 3 g/l, Sodium citrate dihydrate 0 5 g/l, $MgSO_4$ 7 H_2O 0 1 g/l, $(NH_4)_2SO_4$ 1 g/l, glucose 2 g/l
5 KH_2PO_4 6 g/l, K_2HPO_4 1 4 g/l, Sodium citrate dihydrate 1 g/l, $(NH_4)_2SO_4$ 2 g/l, $MgSO_4$ 7 H_2O 0,1 g/l glucose 5 g/l, 1 glutamic acid 50 mg/l, 1 asparagine 50 mg/l
6 K_2HPO_4 6 g/l, KH_2PO_4 1 4 g/l, Sodium citrate dihydrate 1 g/l $MgSO_4$ 7 H_2O 0 1 g/l, $(NH_4)_2SO_4$ 2 g/l, glucose 5 g/l, 1 glutamic acid 50 mg/l, 1 asparagine 50 mg/l, Casamino acids (Difco) 0 2 g/l Yeast Extract (Difco) 1 g/l
7 Bacteriological peptone (B p) (Oxoid) 10 g/l, NaCl 5 g/l
8 B p 10 g/l, NaCl 5 g/l, glucose 2 g/l
9 B p 5 g/l, NaCl 5 g/l, Yeast Extract (Difco) 5 g/l
10 B p 5 g/l, NaCl 5 g/l, Yeast Extract (Difco) 5 g/l, glucose 2 g/l
11 Brain Heart Infusion (BHI) (Oxoid) 37 g/l
12 BHI (Difco) 37 g/l

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Preparation of Dialysed Medium

Twice the regular concentration of solutes was dissolved in doubly distilled water and dialysed 14-18 hours against an equal volume of water at 4° C The dialysate was autoclaved and used as growth medium

radiation is now in progress in order to see whether there is a relation between dose rate and inactivation effect

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highest yield of phospholipase C was obtained in media [redacted] and 10, both containing yeast extract The yield was about 20 per cent lower in medium 2, but this medium contained no macromolecules and was therefore convenient when phospholipase C was to be purified In most cases no enzyme activity was found when *B cereus* was grown for up to 10 hours in BHI (Oxoid) medium, with or without the macromolecular part of the medium (Table 1) BHI (Difco) gave normal growth and enzyme activity

TABLE 1 *Phospholipase C Production by* B cereus *in Various Media*

Medium	Growth of B cereus	Highest enzyme activity (units/O D $_{600}$)
1	+	13 0
2	+	13 5
3	—	0
4	—	0
5	—	0
6	+	1 1
7	+	11 0
8	+	12 7
9	+	16 5
10	+	17 7
11	+	0
12	+	13 1

Inhibition of Phospholipase C Synthesis

B cereus was grown in a mixture of varying amounts of BHI (Oxoid) and N b dialysate Aliquots were withdrawn each hour and tested for growth and activity The enzyme appeared later and the activity increase levelled off earlier when increasing amounts of BHI was added to the medium (Fig 2) Cells harvested from such medium contained less intracellular phospholipase C activity than cells grown in N b dialysate No inhibition

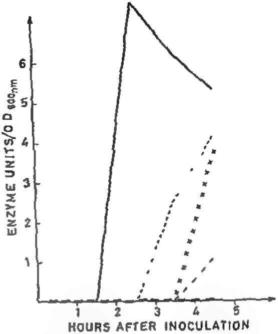

Fig 2 Phospholipase C activity in media with increasing BHI content
N b medium ———
BHI N b (1 3) – ––
BHI N b (1 1) x x x x x
BHI N b (3 1) –
BHI - - - - -

TABLE 2. B cereus Grown in Mixtures of Calf Brain or Beef Heart Extract and Nutrient Broth Dialysate
Phospholipase C activity (units/O D $_{600}$)

Medium	Incubation time (hrs) 1	2	3	4	5	6
Nutrient broth dialysate	0	[redacted]	17 0	16 0	16 0	15 7
Nutrient broth dialysate Calf brain extract 3 1	[redacted]	0	15 8	16 0	16 2	16 4
Nutrient broth dialysate Calf brain extract 1 1	0	0	14 6	15 9	19 9	16 4
Nutrient broth dialysate Calf brain extract 1 3	0	0	7 7	15 3	15 8	16 9
Nutrient broth dialysate Beef heart extract 3 1	0	0	15 8	16 2	13 3	14 0
Nutrient broth dialysate Beef heart extract 1 1	[redacted]	0	5 4	9 2	0	0
Nutrient broth dialysate Beef heart extract 1 3	0	0	0	0	0	[redacted]

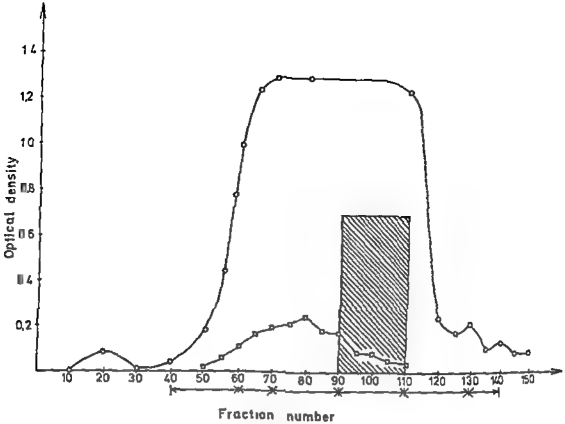

Fig 3 Filtration through Bio Gel P 10 of lyophilized and reconstituted dialysate of beef heart extract. The fractions indicated were pooled, concentrated and tested for their inhibitory activity
OD_{450} ○—○ OD_{360} □—□

was observed when new or used BHI medium was mixed with enzyme solution The absence of any activity in cultures grown in BHI medium was therefore probably due to an inhibited synthesis To decide if one of the components of the BHI (Oxoid) medium was responsible for the inhibition, various amounts of dialysate of each component was added to nutrient broth dialysate and growth and enzyme activity tested (Table 2) The growth of *B cereus* was the same in all media Addition of beef heart extract to the growth medium inhibited the synthesis of phospholipase C Dialysate of beef heart extract was lyophilized, dissolved in a small volume of water and submitted to filtration through Bio-Gel P-10 Fractions of 2 3 ml were collected and pooled after determination of optical density (Fig 3) Each pool was lyophilized, dissolved in a small volume of water and added to medium 2 Growth and enzyme production in these various media were determined The inhibitory activity was recovered in one of these pools (Fig 3) The inhibitor was not extractable in ether and has not been further identified

Effect of Phosphorylcholine, Phosphorylethanolamine and Phosphorylserine in the Medium

On the assumption that the immediate products of phospholipase C action might repress the synthesis of the enzyme, 1 mM or 10 mM phosphorylcholine phosphorylethanolamine or phosphorylserine was added to medium 2 No inhibition of enzyme synthesis was observed except when growth inhibitory concentrations of phosphorylserine were used

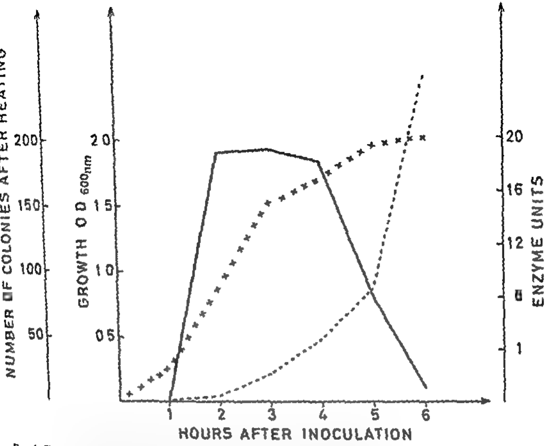

Fig 4 Growth enzyme activity and sporulation in nutrient broth The spores were determined from the number of colonies on BHI agar plates in 0 5 ml medium (diluted 10 ×), after heating 10 min at 80° C Growth x x x x x Enzyme activity ——— Sporulation

Synthesis of Phospholipase C in Relation to Growth and Sporulation

B cereus grown in medium 2 was tested for sporulation and enzyme production The medium was inoculated from a shaker culture grown for 3 hours Hourly aliquots were withdrawn for determination of growth enzyme activity and sporulation (Fig 4) The highest enzyme activity appeared at 2-4 hours and declined before the rise in heat resistance Sporulation was observed in cultures of medium 11 (no enzyme activity) and medium 10 (maximum enzyme activity) but the sporulation in these media was lower than in medium 2 Colonies of *B cereus* which had been kept on the same BHI plate for a week or more gave rise to cultures with reduced ability to sporulate whereas the growth and enzyme activity of such colonies were normal

DISCUSSION

Phospholipase C was found in the culture filtrate of *B cereus* (1 3) Very little enzyme activity was detectable inside the cells, confirming that phospholipase C is an exoenzyme The dialysate of nutrient broth was a very convenient medium when purification of phospholipase C was to be undertaken

In most cases *B cereus* grown in BHI (Oxoid) medium (medium 11) had no phospholipase C activity within or outside the cells This could be due to inhibition of enzyme synthesis or of enzyme activity No loss

of enzyme activity was observed when the enzyme was incubated with used or fresh BHI medium The enzyme was therefore probably repressed rather than inhibited by some factor of the BHI (Oxoid) medium This factor was dialysable, heat stable and insoluble in ether

The role of phospholipase C in bacterial physiology is not understood The enzyme activity decreased before the amount of spores increased when *B cereus* was grown in medium 2 Sporulation was observed in media which allowed phospholipase C synthesis (medium 2 10) as well as in inhibitory media (medium 11) The number of heat resistant, colony forming spores was low when media 2, 10 and 11 were inoculated from agar plates more than 8 days old, whereas growth and enzyme activity were normal There was thus no close connection between the synthesis of phospholipase C and sporulation

Koga & *Kusaka* (6) reported that the enzyme was involved in the process of autolysis of the cell membrane of *B cereus* Cultures of *B cereus* in BHI (Oxoid) medium showed normal growth without enzyme activity even 10 hours after inoculation Phospholipase C is therefore probably not required for normal growth of *B cereus*

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IMMUNOGLOBULIN-CONTAINING CELLS IN THE SMALL INTESTINE IN VIRAL HEPATITIS

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Immunofluorescence microscopy studies of jejunal and hepatic biopsies from 12 patients with viral hepatitis are reported Antisera against IgA IgM, IgG, IgD, IgE, and SP (secretory piece) were employed Significant increase of IgA and IgD containing cells was found in the jejunal biopsies

In recent years it has been suggested that acute viral hepatitis is a disease that affects a number of other organs than the liver However, conflicting data have been presented regarding the stereomicroscopic and light microscopic appearance of jejunal biopsies from patients with acute viral hepatitis This report describes the results of an immunofluorescence microscopy study of jejunal and hepatic biopsies in patients with acute viral hepatitis

MATERIAL

Hepatitis

12 patients aged 15 to 38 years with acute viral hepatitis The diagnosis was based on repeated liver biopsies laboratory results indicating liver cell damage and a typical history

Mononucleosis leptospirosis and collagen disease could be excluded In all cases the clinical course was benign and liver function was normal before discharge from the hospital

All patients were Europeans three had been travelling outside Europe during or shortly before outbreak of the disease Further details of the patients are presented in Table 1

Controls

21 patients aged 19 to 60 years The control patients were admitted to a gastroenterologic department with abdominal complaints but none had important pathological findings after extensive investigations The final diagnoses were obesity irritable colon x ray negative dyspepsia

METHODS

Anti secretory piece (SP) Colostrum collected at the first and second post partum day was used to prepare purified SP as follows The fat was removed by centrifugation at 2-4° C Casein was precipitated by acidification (pH 4 6) with acetic acid After neutralization the β lipoproteins were removed by precipitation with dextran sulphate in the presence of calcium ions The precipitate obtained by making the solution 2 M with respect to $(NH_4)_2SO_4$ [illegible]

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The anti SP was kindly tested by *Lars Aa Hansen* M D Institute of *Medical Microbiology*, University of Gothenburg Sweden, who found reaction of identity with his anti SP antisera

TABLE 1 *Details of Patients with Acute Viral Hepatitis*

Sex	Age	Drug abuse	Australia antigen	Jejunal pathology		Duration of jaundice before biopsy, days
				Stereo micro-scopy	Micro scopy	
♂	20	+	+	+	+	14
♀	16	+	+	—	—	12
♂	38	+	+	—	—	10
♂	19	+	—	—	(+)	21
♂	16	+	+	+	(+)	14
♂	22	—	—	+	+	10
♂	23	—	—	+	(+)	
♂	22	—	—	+	(+)	
♂	19	—	+	—	(+)	20
♀	15	—	+		—	8
♂	22	—	—	—	+	8
♂	23	—	+	—	—	12

tions used to immunize rabbits raised an antiserum which after complete absorption with normal human serum only reacted with free and bound SP when tested against whole colostrum When tested against one of the fractions from DEAE Sephadex chromatography by immunoelectrophoresis a weak reaction with an α_2 component was demonstrated This α_2 component was not due to lactoferrin

Antisera The following antisera were obtained from commercial laboratories FITC conjugated goat anti human IgA and FITC conjugated goat anti human IgM (Hyland, Division Travanal Laboratories, Bruxelles, Belgium), FITC conjugated rabbit anti human IgG (Dakopatts Copenhagen Denmark), FITC conjugated swine anti human IgD (Nordic,
IgE (Pharma
anti human S
gated with FITC as previously described (*Soltoft* 1969) Protein concentration at working dilution was anti IgA 0 4 mg/ml IgM 0 6 mg/ml, IgG 0 2 mg/ml, IgD 1 5 mg/ml IgE 1 5 mg/ml and SP 1 5 mg/ml Optical density ratios (*Brighton et al* 1967) expressing E $_{495}$ E $_{280}$ ranged between 0 3 and 0 8

The following antisera obtained from commercial laboratories were conjugated with tetramethyl rhodamine isothiocyanate Rabbit anti human IgA (Behringwerke, Marburg Lahn Germany), rabbit anti human IgM and rabbit anti human IgG (Dakopatts, Copenhagen, Denmark) Protein concentration on working dilution was 0 4–0 5 mg/ml Optical density ratios expressing E $_{515}$ E $_{280}$ ranged between 0 11–0 22

Each conjugate was absorbed with guinea pig liver powder 100 mg powder per ml conjugate Thiomersol to 1 10 000 was added to the conjugates Only FITC conjugated antisera were used for quantitation the same lots being used throughout this study

Working dilution All conjugates were tested in serial dilutions on serial sections of jejunal biopsies to determine the optimal staining titre The plateau end point of titration was determined as the first dilution which caused reduction in the number of fluorescent cells The working dilution was 2 to 4 times more concentrated than the "end point" dilution Anti IgG was also tested in lymphnode in which it was easier to identify the end point Anti IgD was in addition tested on tonsil because of its high density of IgD-containing cells

Control of antisera By immunoelectrophoresis the antisera against human IgA IgM IgG and IgD gave only one line at the expected place using as antigens normal whole human serum and serum samples containing homologous nominally monoclonal globulins of the different classes Immunoelectrophoresis with non corresponding myeloma protein, kappa and lambda Bence Jones protein at different concentrations gave no precipitation Each fluorescent antiserum could be neutralized by absorption with a corresponding myeloma serum Blocking tests were performed *It was* possible to block the specific staining *of the* antisera by preincubation of the sections with unconjugated homologous antisera, whereas *pre-incu*bation with unconjugated non *homologous* anti

The immunoelectrophoretical analyses and the preparation of anti secretory piece were kindly performed by *B Mansa* Biophysical Department, Statens Seruminstitut Copenhagen, *Denmark*

sera did not block. However, complete blocking was only obtained with undiluted unconjugated antisera followed by 20 fold diluted conjugated antisera.

Finally the specificity was tested on bone marrow samples from patients with IgG or IgA myeloma (Fig. 1) or on splenic sections from a patient with Waldenstrom's macroglobulinaemia. Cross reactivity was further excluded in sequential staining experiments. This was regularly done with anti IgD as no tissue from a IgD myeloma patient was available. The anti IgE satisfied the above criteria and against the IgE test solution provided by the manufacturers a single precipitin line was formed.

Biopsy specimen material. The jejunal mucosal biopsies were obtained with a peroral multiple biopsy tube. All specimens used for quantitation were taken at or 10 cm distal to Treitz' ligament. Though the localization was controlled by fluoroscopy minor variations could not be excluded. To evaluate this 2 biopsies were taken at a distance of 10–15 cm at the level of Treitz' ligament. In 10 patients quantitation of IgA and IgM containing cells were carried out, but no differences between the higher and the lower biopsies could be demonstrated.

Biopsies for immunofluorescent studies were immediately frozen and stored over dry ice. Storage over dry ice for as long as 3 years did not destroy the staining properties of jejunal biopsies. Liver biopsies were obtained by the Menghini technique. Serum immunoglobulin levels were determined according to *Weeke* (1968). The serum samples were obtained at the same time as the biopsies.

Sectioning. 4 μm serial sections were made with a cryotome. The thickness of the sections was checked by the method of *Brattegaard* (1953) using a microscope focused on upper and then lower surface of the preparation and measuring the difference with a micrometer. With a setting of the microtome on 4 μm the thickness was 4.1 μm ± 0.3 μm (95 per cent range). In sections of rectal and ...

... at 22° C for 10 minutes was carried out. It was important to avoid drying of the section at any stage.

Comparison of the following fixation procedures was made: acetone at 4° C or 22° C for 3, 10, 15 or 20 minutes; 96 per cent alcohol at 4° C or 22° C for 3, 10, 15 or 20 minutes; acetic acid alcohol solution at −20° C for 15 minutes; absolute methanol at 4° C or 22° C for 3, 10, 15 or 20 minutes; paraffin embedding method (*Sainte Marie* 1962). Fixation in acetone for 10 minutes at 22° C was chosen as it gave the best contrast between the cytoplasm and the surrounding ground substance.

Washing. After fixation the slides were washed in phosphate buffered saline (0.01 M phosphate, pH 7.2) for 15 minutes.

Stirring, longer washing, or repeated washing seemed not to be necessary. The pH of the buffer was altered within a wide range (pH 5–9), but no effect on the final preparations could be detected. Whether barbitone buffer, phosphate buffer, or saline were used at this step (and at washing after staining) seemed to be of minor importance.

Staining

Single incubation. The slides were covered with one drop of the conjugate diluted to the working titre and incubated for 30 minutes at room temperature. After incubation the slides were washed in three successive baths of the phosphate buffer for 15 minutes and mounted with buffered glycerine (50 per cent). The slides were kept in darkness at 4° C and studied within 40 hours after the staining.

Incubation time seemed not to be critical because incubation in the range 10–120 minutes did not influence the result, neither did alterations in incubation temperature between 4° C and 22° C. Shorter washing time left some unspecific staining. Other mounting media, as Eukitt (Kindler, Freiburg, Germany) or buffered glycerine (90 per cent) were also satisfying.

Double incubation. In blocking tests a second incubation with an appropriate conjugate was carried out when the slides had been washed after the first incubation.

Sequential staining. In control experiments sequential staining was carried out to examine whether cross reactions took place. A section was studied after staining with one FITC antiserum. The fluorescence was bleached by exposure to the unshielded mercury lamp. A second staining of the same section with another FITC antiserum could then be carried out. To get complete fading at least 10 minutes exposure was necessary.

Microscopy and measurement. A Leitz Orthoplan microscope with a Super Wide immersion darkfield condensor (Tiyoda) was used. The microscope was equipped with an Osram HBO 200 W high pressure mercury lamp, a Philips 100 W Halogen lamp and a 60 W Tungsten lamp. A FITC interference research filter with red contrast band was used as primary filter and a 515 nm filter as secondary filter (*Rygaard & Olsen* 1969). With tetramethylrhodamine isothiocyanate as fluorochrome a 546 nm interference primary filter and a 610 nm secondary filter was used (Optisk Laboratorium, Lyngby, Denmark).

Quantitative measurements of immunoglobulin containing cells were obtained by planimetry. As

the immunoglobulin containing cells were confined to the interstitial tissue they were quantitated by relating the fluorescent area to the total area of the interstitial tissue When quantitation was carried out the microscope was equipped with a Leitz drawing aggregate This equipment permitted simultaneous microscopy and drawing The drawing was done with a white pencil on a black sheet of paper to reduce the light coming into the microscope The areas to be measured were cut out and weighed This procedure was less time consuming than previous ones and permitted quantitation of the complete section and selection of visual fields could be avoided

Biopsies of different size were accepted for quantitation if more than 10 visual fields were available for quantitation Sections consisting almost completely of either villi or crypts were excluded

The microscope was equipped with a Leitz photo automate, Ilford FP4 black white film or Anscochrome 28 DIN daylight film were used The red contrast band from the FITC interference filter was eliminated with a BG23 filter, when photomicrographs had to be taken

Reproducibility Reproducibility was studied on several sections of normal and pathological jejunal biopsies Quantitation of IgA and IgM containing cells revealed a coefficient of variation of less than 0 17 No special precautions were taken to eliminate observer bias but it should be kept in mind that the results of quantitation are calculated from planimetry of the interstitial tissue and the specific fluorescent area in more than 10 different and complicated visual fields

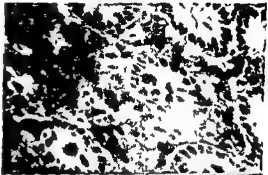

Fig 1 Fluorescence of IgA myeloma cells from bone marrow stained with FITC anti human IgA

Fig 2 IgM-containing cells in the crypt area of jejunum from a control patient

Fig 4 Jejunal biopsy stained with FITC anti human secretory piece Fluorescence of epithelium cells is seen No fluorescence of interstitial tissue or goblet cells

RESULTS

Controls

With IgA the expected predominance of cells containing this immunoglobulin was found In addition to the fluorescent cells in the lamina propria, fluorescence of the luminal end of epithelium and crypt cylinder cells was seen whereas the goblet cells were unstained There was no nuclear staining with this or any of the other antisera With anti IgM no epithelial staining was seen The fluorescent cells were fewer but a little larger than the cells containing other types of immunoglobulin (Fig 2) The IgG containing cells were difficult to detect because of the strong interstitial fluorescence produced in the intestine This fluorescence is probably due to IgG in the intercellular fluid and is much stronger than in other tissues where IgG containing cells were easily recognized (Fig 3) The IgD containing cells were almost as numerous as IgG containing cells in the jejunal biopsies Epithelial structures were not stained with this antiserum The few IgE containing cells gave a good contrast against the background However, IgE cells were only found in half of the patients even when several sections were screened and

3

5

Fig 4 IgG containing cells in a section of human lymphnode stained with FITC anti human IgG

Fig 5 Human jejunal biopsy stained with FITC anti human IgM The interference filter gives yellow green fluorescence and a red background staining

when present only a very limited number was seen

With anti-SP, staining of the crypts and surface epithelium cells was obtained (Fig 4 The immunofluorescence was strongest at the luminal end whereas neither nuclei nor goblet cells were stained The staining was even throughout a section and no part of the epithelium was unstained The gastric, colonic, and rectal mucosa showed identical stain-

ing of the epithelium. In other organs no specific staining could be recognized, but an unspecific fluorescence of collagen fibrils was seen.

The cells giving specific staining with the five antisera directed against each of the immunoglobulin classes were rather similar. The majority was plasma cells, but a few had features more typical of lymphocytes. Fluorescent cells in the epithelial layer were seldom encountered. Clusters of stained cells were frequently seen with anti IgA and sometimes with anti IgM.

With the FITC research interference filter the contrast between the green fluorescence and the red background made the interpretation easy (Fig. 5). With this filter combination, eosinophilic granulocytes and mast cells were easily recognized because of the bright red granular fluorescence. They were not mistaken for specifically fluorescent cells as might happen with other optical systems.

Quantitation was carried out by relating the area of cells giving specific immunofluorescence with a given antiserum to the area of the complete interstitial tissue, and the results were expressed as per cent. The following mean value and standard deviations (2 SD) were found: IgA, 11.3 ± 5.2, IgM 3.9 ± 2.6, IgG 1.8 ± 1.2, IgD, 1.3 ± 1.0, IgE less than 0.1.

Hepatitis

Liver biopsies from 10 of the patients were stained with fluorescent antisera against the five immunoglobulin classes. The immunoglobulin containing cells were only occasionally seen outside the portal tract. IgA containing cells were seen in all biopsies but were only slightly more numerous than IgG cells. IgM cells were only seen in 5 biopsies. IgD and IgE cells in none.

Jejunal biopsies. The stereomicroscopic appearance was abnormal in five patients whose villi were shortened. In one of the patients small spots with more pronounced villus atrophy were seen. Light microscopic examination revealed a heavy cellular infiltration of lamina propria in three cases and another five patients presented a slight increase of interstitial cells.

Immunofluorescence microscopy revealed increased number of immunoglobulin containing cells, yet the normal pattern with IgA > IgM > IgG > IgD > IgE was maintained. Quantitation of the fluorescent area revealed the following mean values and standard deviations (2 SD): IgA 14.6 ± 4.8, IgM, 4.5 ± 3.8, IgD, 2.3 ± 1.4, the number of IgG cells appeared normal but was not quantitated. The IgE cells were few as usual. The number of IgA cells was significantly increased ($P < 0.01$, Fig. 6) as was the number of IgD cells ($P < 0.01$, Fig. 7) when compared with the controls (Wilcoxon rank sum test). The IgM containing cells were found in normal numbers.

The majority of fluorescent cells was plasma cells, but an increased number of lymphocyte like cells were seen. The Ig cells did not penetrate the epithelium to a greater extent than usual and the highest density of Ig cells was found among the crypts. Anti SP stained the same structures as in the control group.

No differences were found between Au

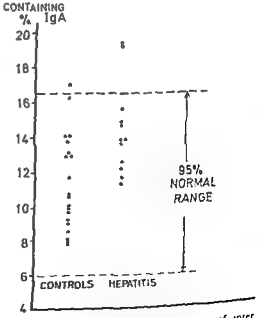

Fig. 6. IgA containing cells as per cent of interstitial tissue in jejunal biopsies from controls and from patients with viral hepatitis.

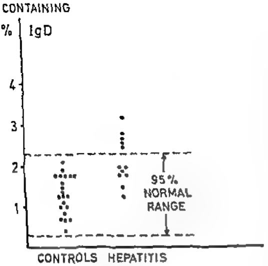

Fig 7 *IgD*-containing cells as per cent of interstitial tissue in controls and in viral hepatitis

stralia antigen positive and negative patients or between patients with admitted drug abuse and the rest of the patients

Quantitation of serum immunoglobulins in patients with hepatitis revealed the following mean values IgG 15 5 g/l (95 per cent range 7 2 15 1 g/l), IgA 1 39 g/l (95 per cent range 0 74–3 06 g/l), and IgM, 1 34 g/l (95 per cent range 0 23 1 33 g/l)

DISCUSSION

The first study of jejunal biopsies in viral hepatitis was reported by *Astaldi et al* (1964) who found a variety of pathological changes from short stumpy villi to a completely flat surface The biopsies were taken at different levels of the intestine and at different stages of the disease In their study on US soldiers in Korea *Conrad et al* (1964) similarly found an almost complete loss of villi in prolonged cases *Kudzma et al* (1969) studying soldiers in U S A found no pathological changes in biopsies from the proximal jejunum These discrepant results might be explained by environmental factors, as it is known that mucosal abnormalities are frequent in the East Asian area (*Lindenbaum et al* 1966) The stereomicroscopic abnormalities found in our study were mild and only seen in a few patients, even when the biopsies were obtained during maximal disease activity

Light microscopic examination revealed increased infiltration with mononuclear cells in lamina propria Similar findings have been experienced in Italian adults (*Astaldi et al* 1964), German children (*Mietens & Vogel* 1971), American soldiers in U S A (*Sheehy et al* 1964), or in Korea (*Conrad et al* 1964) Besides, other pathological changes ranging from thickening of villi to complete villus atrophy were found in these studies *Kudzma et al* who did not find any abnormalities were the only investigators who included control material and evaluated the biopsies at random

The results of immunofluorescence microscopy of liver biopsies are in accordance with those of *Hadziyannis et al* (1969) We did not attempt to quantitate the immunoglobulin containing cells but the majority belonged to the IgA-class

The major findings in the immunofluorescence microscopy of jejunal biopsies from control patients are in accordance with a previous report from this and other laboratories (*Crabbe* 1965, *Soltoft* 1969) The quantitative measurements gave somewhat lower values than previously This was to be expected as the calculations now are based on planimetry of a complete section and not on a limited number of visual fields selected because of richness in Ig cells The IgD cell quantitation showed them less numerous than IgA IgM , and IgG cells as reported by others (*Crabbe & Heremans* 1966, *Rowe et al* 1968) However, the absolute number of IgD cells was somewhat higher in our material Despite the limited number a relative predominance of IgD has been found, as the IgD cells are several times more numerous than could be expected from the serum level of IgD This is in accordance with the results published by *Rowe et al* (1969) where a similar predominance was present, although it was not mentioned by the authors

Immunofluorescence microscopy has de-

monstrated a significant increase of IgA- and IgD containing cells in viral hepatitis It is surprising that the IgM- and IgG containing cells show no variations, as an increase might be expected according to the increase in the serum level of IgG and IgM in this condition Our study is in accordance with several other studies where it has been found that the hypergammaglobulinaemia in viral hepatitis is due to increase of IgG and IgM, and to a much lesser degree of IgA (*Wollheim* 1968, *Lee* 1965, *Jensen* 1970) Subnormal serum IgD has been found in this condition (*Bachmann* 1967) The discrepancy between alterations in serum immunoglobulins and the changes in the jejunal mucosa illustrate that the blood immunoglobulin level is a poor indicator of the mucosal immunoglobulin state

That a disease affecting the intestinal mucosa may be accompanied by increase of the IgA containing cells seems reasonable The locally produced antibodies in a number of viral and bacterial infections have been proven to be of IgA type (*Ogra et al* 1968, *Kasel et al* 1969) This is also likely to be the case in viral hepatitis

The increase of IgD-containing cells described in this study is of special interest Until now increase of IgD cells has only been described in one condition also affecting the gastrointestinal tract *Crabbe* & *Heremans* (1966) described a pronounced IgD cell infiltration in rectal biopsies in a single case of ulcerative colitis However in six additional cases and in other series (*Gelzayd et al* 1968) no IgD increase has been found The increase of IgD cells in the intestinal mucosa during viral hepatitis the relatively high density of IgD cells in the mucosa and in adenoid tissue are suggestive of a relationship between IgD and the external surfaces

This study was supported by grants from *Statens lægevidenskabelige Forskningsråd*

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15 [illegible] Nahrungsproteine und Veränderungen der

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THE GROWTH AND MORPHOLOGY OF *ACHOLEPLASMA (MYCOPLASMA) LAIDLAWII A* IN DIFFERENT MEDIA

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In the present study we have tried to find optimal cultural conditions for rapid growth and large yields of the actively growing A laidlawii A cells The earlier findings on the growth promotion of oleic and elaidic acids were confirmed The modified Edward and Butler & Knight medium was superior to all other media tested when colony forming units were used as a measure of growth The highest yields expressed as a total cell protein were obtained both in the elaidic acid—tryptose broth and in the Edward medium after 24 hours' growth The cells grown in the basal tryptose broth appeared in the electron microscope as large (Ø 600–1000 nm) spherical bodies throughout the growth cycle The addition of unsaturated fatty acids induced A laidlawii A to divide and to multiply as smaller (Ø 300–500 nm) round bodies connected with the active growth On the basis of earlier findings and the present results it is suggested that the multiplication of A laidlawii A occurs by division

Numerous investigations have been made on the cultural requirements of the Mycoplasmatales (*Rodwell* 1969, *Sharp* 1970) In view of the lack of a defined medium suitable for all mycoplasma species, the growth conditions have, however, usually been modified according to the purposes of the study, resulting in variable yields and cell morphology of the organisms (*Freundt* 1969, 1970, *Anderson* 1969)

During our studies of nucleotide metabolism of *Acholeplasma (Mycoplasma) laidlawii A* (*Virkola* 1970) it was found that the total amount of free nucleotides of this organism is relatively small We therefore tried to find optimal cultural conditions for rapid growth and large yields of the actively growing *A laidlawii A* in the modified *Edward* and *Butler & Knight* (1960) medium (*Virkola* 1971) On the other hand it is known that the addition of fatty acids to a basal tryptose broth medium improves the growth and formation of filaments in mycoplasma (*Razin et al* 1966, *Rottem & Panos* 1969) We therefore compared the effect of different media on the growth and morphology of *Acholeplasma laidlawii A*

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MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were of analytical grade

Organism

Acholeplasma (Mycoplasma) laidlawii A was kindly donated by *E A Freundt*, Institute of General Pathology and Bacteriology, University of Aarhus, Denmark

Cultural Conditions

Medium I was modified from the medium of *Edward* (1947) and *Butler & Knight* (1960), *Virkola* (1971)

Medium II Basal tryptose broth (pH 8 0) (*Razin et al* 1966) to which fatty acids were added in ethanolic solutions in the following final concentrations a) oleic 5 µg/ml, b) oleic 50 µg/ml, c) elaidic 5 µg/ml and d) elaidic 50 µg/ml (Sigma) The final concentration of ethanol in the medium was below 0 5 per cent (v/v)

The adaptation of *A laidlawii A* to medium II and the growth experiments were performed according to *Razin et al* (1966) The size of the inoculum was 0 5 per cent taken from a 24 hour old (achole)mycoplasma culture During the study the strain was kept alive by daily subcultures Any bacterial contamination was controlled on blood agar plates All growth experiments were performed at 37° C Because of the artificial aggregation of the culture medium I lipoproteins during agitation (*Virkola* 1971) only static cultures were used

Measurement of Growth

The living cells were estimated by the colony count method for mycoplasma (*Butler & Knight* 1960) the diluent was medium I which was also solified for plates with agar to a final concentration of 0 9 per cent (w/v)

For cell protein measurements, 45 ml culture samples were collected according to *Razin et al* (1966) and the protein was determined as total nitrogen (micro Kjeldahl) protein expressed as 6 15 × N

Electron Microscopy

The organisms were prefixed for electron microscopy studies as follows 100 ml culture samples, taken at 6, 9, 12, 25, 40, 48 hours, were added to 100 ml 12 5 per cent glutaraldehyde (*Maniloff* 1970) in 0 2 M phosphate buffer, fixed for [illegible] hours at +2° C and separated at 14,000 × g(10 min, +2° C)

After fixation the cells were washed and stored in 0 2 M sucrose—0 2 M phosphate buffer until further use For electron microscopy the cells were fixed (*Anderson & Barile* 1965) for 60 min with 1 per cent OsO_4 at +4° C, dehydrated through progressive ethanol series and embedded in Epon 812 (*Luft* 1961), sectioned with a Porter Blum microtome and double stained with 1 per cent uranyl acetate and lead citrate (*Frasca & Parks* 1965) or with lead citrate alone (*Reynolds* 1963) Electron micrographs were taken with a Siemens Elmiskop 1A microscope at original magnifications from 10 000–40,000

RESULTS

The growth of *Acholeplasma (Mycoplasma) laidlawii A* in different media measured as colony forming units and total cell protein is shown in Table 1 The Edward medium (Medium I) was shown to be superior to all other media tested when colony forming units

TABLE 1 *Growth of* Acholeplasma laidlawii A *in Different Media*

Medium	Age of culture				
	12 h	24 h	53 h	49 h	24 h
	colony forming units/ml			pH	mg cell protein/ml
Basal tryptose broth	4 0 × 10^9	3 4 × 10^7	5 3 × 10	7 7	3 9
+ oleic acid 5 µg/ml	4 0 × 10^9	6 0 × 10^8	1 0 × 10^8	7 4	8 2
+ oleic acid 50 µg/ml	4 8 × 10	6 9 × 10^7	0	6 8	4 7
+ elaidic acid 5 µg/ml	1 7 × 10^9	1 5 × 10^9	8 0 × 10^3	6 8	12 8
+ elaidic acid 50 µg/ml	1 0 × 10^5	2 9 × 10^9	2 1 × 10	6 3	14 2
Edward Butler Knight (modif)	2 6 × 10^9	1 6 × 10^9	0	5 8	14 2

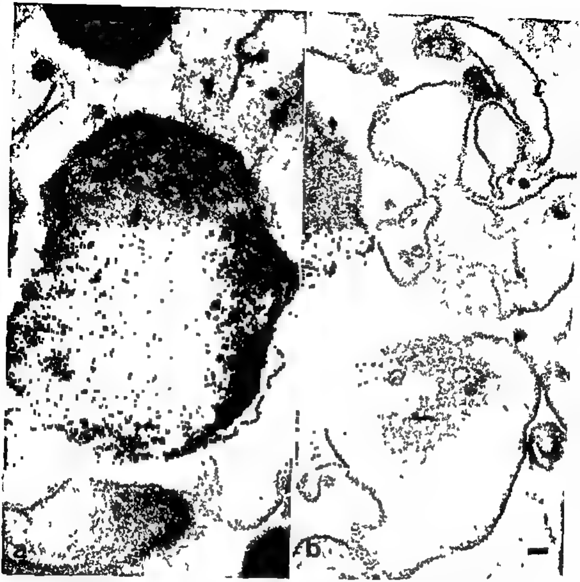

Fig 1 Sectioned *A laidlawii A* cells grown in basal tryptose broth a) after 25 hours b) after 48 hours The cells appear during the active growth as large spherical bodies (1a), which become empty at the end of the growthcycle (1b) Bar markers in this and all other micrographs represent 100 nm × 60 000

were used as a measure of the growth This was expressed both as a shorter lag phase and a higher cell yield

A sharp decline in the colony forming units accompanied by a drop in pH was found after 53 hours growth in the medium reinforced with 50 μg/ml of oleic or elaidic acid, as well as in the Edward medium The highest yields measured as total cell protein in 100 ml medium were obtained in basal tryptose broth supplemented with elaidic acid and in Edward medium after 24 hours growth

The cells grown in the basal tryptose broth appeared as large (Ø 600–1000 nm) spherical bodies (Fig 1a) throughout the growth cycle even when empty apparently nonviable cells appeared at the end of the growth (*Fig 1b*)

The better the organisms grew, the earlier the large cells were overruled by smaller cells

(Ø 300 500 nm) in the cultures containing oleic or elaidic acid With 5 µg/ml fatty acid in the medium small dividing cells seemed to appear in the culture after 6–9 hours whereas with 50 µg/ml of fatty acid small cells were not found for 12 hours

A relatively homogenous population of small cells was observed in the cultures containing 5 µg/ml oleic and 5 or 50 µg/ml elaidic acid after 24 hours (Fig 2) With 50 µg/ml of oleic acid, the population of the same age was still composed of several large cells (< 1000 nm) among the smaller bodies

Towards the end of the growth cycle (48 hours) the cells from the best growing fatty acid culture (elaidic acid 5 µg/ml) were dense and spherical whereas the organisms of other fatty acid cultures clearly had a degenerative appearance with small round spherules or elementary bodies (60–100 nm), (Fig 3)

The addition of fatty acids to the medium did not promote filamentous growth at any stage and only a few filament like cells up to 2 µm long were found in the early growth phase

In the Edward medium the dividing cells (Ø 200–400 nm) appeared in the population even earlier (6 hours) than in the cultures with fatty acid after which the cells were homogenous in size During the first 24 hours many cells were slightly elongated (Fig 4) without any real filament formation Towards the end of the growth (40 hours) the cells turn more spherical (Fig 5a) and irregular vacuolar cells accompanied by many small elementary bodies were finally (48 hours) observed (Fig 5b)

Independent of the growth medium the cells had a typical triple layered unit membrane Several dense small (about Ø 50 nm) round protrusions surrounded by the membrane

Fig 2 A laidlawii A grown for 12 hours in the basal tryptose medium with added 5 µg/ml elaidic acid A relatively homogenous population of small and dividing cells is observed Large granules can be seen inside of the cells × 60 000

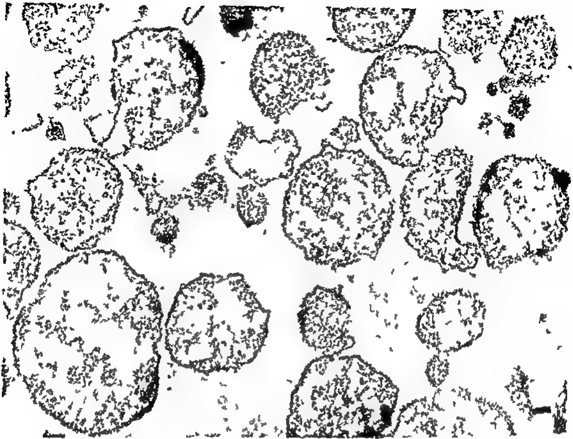

Fig 3 Degenerative cells of *A laidlawii A* after 48 hours growth in the basal tryptose medium reinforced with 50 µg/ml oleic acid Pouches of the membrane of the cells are partly empty and small round spherules elementary bodies , are observed × 60 000

brane were found in the cells grown in the Edward medium (Fig 4) In the cells grown in other culture media the part of the pouches of the membrane were partly empty (Figs 2 and 3)

In the cytoplasma fibrillar areas, probably the nuclear material of the cell granules of various sizes and densities were found Large granules were detected in the different tryptose broth cultures, especially during the early hours of growth (Fig 2)

DISCUSSION

The Fatty Acids as Growth Promotors

The mycoplasma cell has a lipoprotein membrane containing virtually all the cellular lipids (*Razin 1967, Smith 1970*) Some mycoplasmas are capable of synthetizing long chain saturated fatty acids from acetate (*Rottem & Razin 1967, Pollack & Tourtellotte 1967*) while some species require an exogenous octadecenoic acid for growth (*Razin & Rottem 1963 Razin et al 1966 Rodwell & Peterson 1971*) So far all mycoplasmas studied have been found capable of incorporating both saturated and unsaturated fatty acids from the medium into their membrane lipids (*Razin et al 1966 Rottem & Razin 1967*)

Razin et al (1966) have found that increasing the ratio of unsaturated to saturated fatty acids in the medium causes *Acholeplasma (Mycoplasma) laidlawii B* to grow as long filaments accompanied by a marked increase in the cell yield Good growth was obtained with several different unsaturated fatty acids The optimal concentration was about 50 µg/ml, whereas higher concentrations are

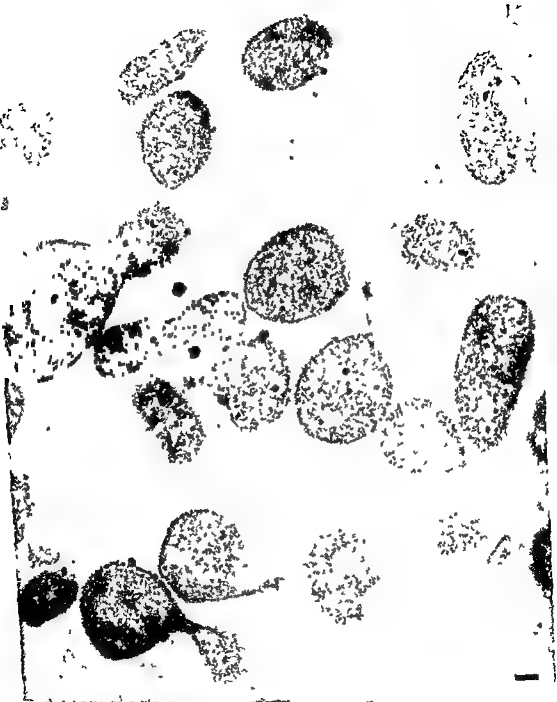

Fig. 4. Small dividing cells of *A. laidlawii A* after 12 hours' growth in the modified Edward-Butler-Knight medium. Several dense, round protrusions surrounded by the membrane are found in the cells. × 60 000.

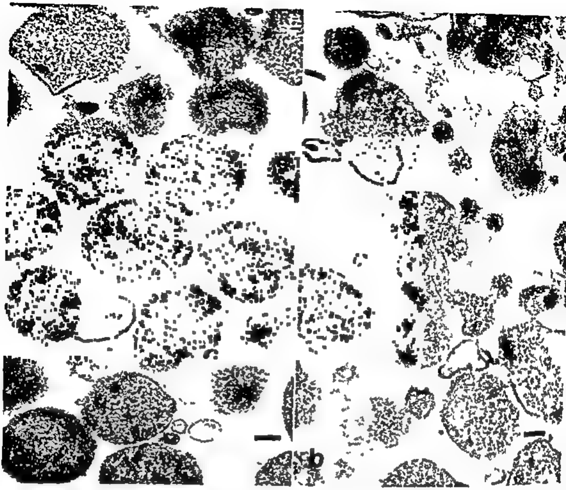

Fig 5 *A laidlawii A* grown in the modified Edward-Butler Knight medium a) after 40 hours, b) after 48 hours Mostly spherical cells are observed (5a), which finally turn to irregular, vacuolar cells (5b). Both samples are double stained

creased the yield In contrast, *Henrikson & Panos* (1969) have reported that long-chain fatty acids do not improve the growth of *A laidlawii B* They explained this discrepancy in the results by the composition of the growth media

On the other hand, *A laidlawii A* grew in a tryptose medium only after the addition of long-chain fatty acids (*Rottem & Panos* 1969) The organism preferred elaidic to oleic acid Elaidic acid was also tolerated in higher concentrations than oleic acid under otherwise identical conditions

In our studies, where *A laidlawii A* was grown in a basal tryptose broth, the effects of fatty acids were similar to those observed by *Rottem & Panos* (1969) The optimal concentration of oleic and elaidic acids with A laidlawii A was about 5 $\mu g/ml$ The growth was markedly better with elaidic than with oleic acid Our modification of the Edward and Butler & Knight medium seemed, however, to provide more favourable conditions for rapid growth than the basal tryptose broth supplemented with fatty acids

The Morphology and the Multiplication of A laidlawii A

The cell morphology and cell multiplication of mycoplasmas are, for the most part, still controversial (*Freundt* 1969, 1970, *Anderson* 1969) The reasons for this are based upon

the apparent ease with which many, if not all (*Maniloff* 1969, *Boatman* & *Kenny* 1970), mycoplasmas can change their cellular morphology with the growth conditions and the subsequent procedures employed to observe the cells

We found that the variation in growth in the different media was also reflected in the morphology of *A laidlawii A* The cells grown in the basal tryptose broth were large, spherical bodies during the entire growth cycle The addition of unsaturated fatty acids induced the organisms to divide and to multiply as smaller bodies Excess oleic or elaidic acid delayed the appearance of smaller cells In the modified Edward medium, the small cells appeared even earlier than otherwise seen when elaidic acid was added to the basal tryptose broth The actively growing cell population in the Edward medium was homogenous in size (Ø 200–400 nm) and form

It has been suggested that the multiplication of these organisms occurs

a) by the development of "elementary bodies within the filaments and by subsequent release of these structures through fragmentation of filaments as demonstrated particularly with *M mycoides* (*Freundt* 1958, 1969 1970)
b) by the development of multiple 'elementary bodies' within the cytoplasm of larger nonfilamentous cells and their subsequent release by disintegration or segmentation of the cell (*Klieneberger-Nobel* 1962)
c) by division as demonstrated with M gallisepticum (*Morowitz* & *Maniloff* 1966, *Maniloff* & *Morowitz* 1967) with *M orale* and *M pneumoniae* (*Furness* 1970) and with *A laidlawii B* (*Maniloff* 1969)
d) by budding (*Liebermeister* 1960, *Anderson* 1965 1969, *Dutta et al* 1965)

In our experiments 'elementary bodies' of *A laidlawii A* appeared only during the late degenerative phase Hence, it is not likely that propagation proceeds by the development of multiple elementary bodies' within large cells

The small protrusions surrounded by a membrane which were visible during the whole growth cycle suggest that *A laidlawii A* could multiply by budding This is contrasted, however, by the fact that no small "elementary bodies' as a result of budding were seen in the cultures before the late degenerative phase Furthermore, *Gourlay* (1971) has reported the presence of a virus of *A laidlawii A* which appears similar to the granules or elementary bodies of the same organism

In our experiments *A laidlawii A* cells were homogenous in size and form during the active growth phase The most likely explanation of reproduction of *A laidlawii A* cells is division

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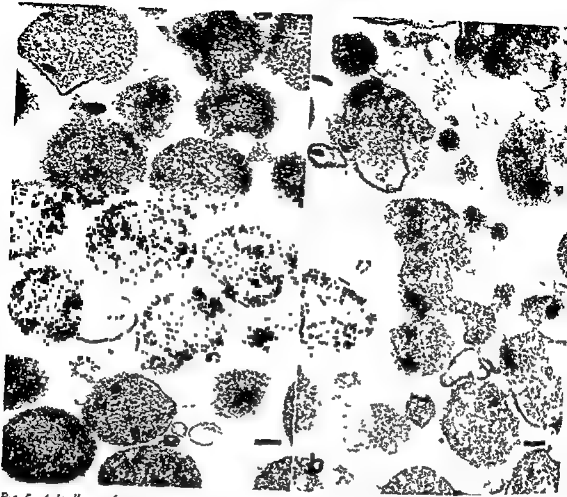

Fig 5 *A laidlawii A* grown in the modified Edward Butler Knight medium a) after 40 hours b) after 48 hours Mostly spherical cells are observed (5a), which finally turn to irregular, vacuolar cells (5b) Both samples are double stained

creased the yield In contrast, *Henrikson & Panos* (1969) have reported that long chain fatty acids do not improve the growth of *A laidlawii B* They explained this discrepancy in the results by the composition of the growth media

On the other hand, *A laidlawii A* grew in a tryptose medium only after the addition of long chain fatty acids (*Rottem & Panos* 1969) The organism preferred elaidic to oleic acid Elaidic acid was also tolerated in higher concentrations than oleic acid under otherwise identical conditions

In our studies, where *A laidlawii A* was grown in a basal tryptose broth, the effects of fatty acids were similar to those observed by *Rottem & Panos* (1969) The optimal concentration of oleic and elaidic acids with A laidlawii A was about 5 $\mu g/ml$ The growth was markedly better with elaidic than with oleic acid Our modification of the Edward and Butler & Knight medium seemed, however, to provide more favourable conditions for rapid growth than the basal tryptose broth supplemented with fatty acids

The Morphology and the Multiplication of A. laidlawii A

The cell morphology and cell multiplication of mycoplasmas are, for the most part still controversial (*Freundt* 1969, 1970, *Anderson* 1969) The reasons for this are based upon

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ANAEROBIC GLIDING BACTERIA ISOLATED FROM THE ORAL CAVITY

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During a 22 day period without oral hygiene 11 dental students rinsed their mouths, twice a day, with a 0 2 per cent aqueous solution of chlorhexidine gluconate The anaerobic bacterial flora of the gingiva was studied and between days 5 and 10 the proportion of gram negative rods increased temporarily and many exhibited a gliding movement of translation Nineteen strains of gliding bacteria were isolated and compared with regard to their colonial and cellular morphology and biochemical characteristics On the basis of these criteria the strains of gliding bacteria are provisionally classified as *Fusobacterium girans* Throughout the experimental period none of the subjects accumulated plaque or developed gingivitis

Based on the bacterial nature of dental plaque and its significance as the prime aetiological factor of both marginal periodontal disease and caries, various agents have been tested as to their ability to inhibit plaque formation (for a review see *Keyes* 1969, *Löe* 1970) Recently, using a human experimental model system the daily use of chlorhexidine gluconate was found to inhibit bacterial colonisation of the tooth surface and to prevent the onset of gingivitis (*Löe & Rindom Schiott* 1970 a, b) and caries (*Löe et al* 1972) This effect was accompanied by a suppression of the salivary flora and by certain changes in the composition of the gingival flora (*Rindom Schiott et al* 1970, *Davies et al* 1970) The most conspicuous change was a temporary increase in the proportion of gram negative rods a large number of which exhibited gliding motility The purpose of the present study was to partially characterise such isolates and consider their classification

MATERIALS AND METHODS

Isolation and Maintenance of Strains

Eleven dental students practised meticulous oral hygiene until the Gingival Index (*Loe & Silness* 1963) and Plaque Index (*Silness & Loe* 1964) approached zero They then stopped toothbrushing and rinsed for 1 minute with 10 ml of a 0 2 per cent aqueous solution of chlorhexidine gluconate at 8 a m and 10 p m daily for a period of 22 days Specimens were taken with sterile swabs from the marginal and attached gingiva adjacent to the upper lateral incisor throughout the experimental period The swabs were immediately placed in Brain Heart Infusion broth (Difco) and after serial 10 fold dilution 0 1 ml of each dilution was

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Fig 2 The cellular morphology of glid ng bacteria after 3 days growth n l qu d plaque med um Phase contrast Magnif cat on 1000 ×

Fig 3 The cellular morphology of gl d ng bacteria after ■ days growth in liqu d plaque medium Ter m nal and subterm nal swell ngs are present in many of the cells Phase contrast Magn f cat on 1000 ×

pH 6 1) and small agar blocks were then fixed o ern ght at room temperature—as descr bed by *Ryter & Kellenberger* (1958 a)—n 1 per cent OsO_4 n the same acetate buffer with the add tion of 1/10 volume YAP med um (yeast extract sod um acetate peptone med um 0 3 0 05 and 0 9 per ce
a br
block 1 per cent uran l acetate n the calcium conta ning acetate buffer The blocks were then dehydrated in alcoh ol and embedded in Ves opal W *Ryter & Kellen be ger* 1958 b) Th ■ sect ons were prepared and po ta ned w th uranyl magnes um acetate (*Frasca & Parks* 1965) and lead citrate (*Reynolds* 1963)

RESULTS

Throughout the 22 day experiment no plaque accumulated and gingivitis did not develop

Although not observed in specimens taken on day 0 gliding bacteria were present in the gingival samples from all 11 students after 5–10 days of chlorhexidine rinsing Subsequently the organisms were either absent or their numbers declined prior to the cessation of the experiment

Nineteen strains of gliding gram negative rods were isolated from 11 samples from 6 students and partially characterised

Colonial Morphology

Colonies of gliding bacteria after 72 hours anaerobic incubation on agar plaque medium were 2–8 mm in diameter and iridescent violet in colour After 7 days incubation the colonies assumed a green colour The periphery of the colonies was ill defined and consisted of islands of growth spreading outwards from the confluent growth centre (Fig 1) All colonies were easily removed and on subculture to agar plaque medium growth comprised a uniform turbidity with some deposit

Good growth of gliding bacteria was obtained on subculture to Trypticase Soy Agar (Baltimore Biological Laboratories), incubated anaerobically but growth on blood agar was poor or absent

Cellular Morphology

The cells of gliding bacteria were gram negative rods with rounded ends (Fig 2) The organisms were 2–20 μm long and 0 2–0 3 μm wide Occasionally very long forms, greater than 50 μm were seen Intracellular granules were observed in most strains and in addition central or subterminal swellings and free cystic bodies 1–3 μm in diameter, were a characteristic finding (Figs 2 and 3) The number of free cystic bodies increased with age of the culture

Ultrastructure

From the negatively stained preparations it was observed that the organism was a long straight rod with blunt rounded ends (Fig 4) It had a cytoplasm of medium electron density Frequently swellings of material with similar electron densities were observed outside the micro-organisms, often in close association with the cell wall (Fig 4) Blebs or strands, possibly of cell wall origin, were also seen extruding from the cell wall (Fig 4) No flagella or appendages such as fimbriae or pili were observed

Similarly, the thin sections revealed long slender rods (Fig 5) The distribution of the cytoplasmic material was typical of gram negative cells but nuclear regions were rather small and indistinct (Fig 7) In addition large areas of the cytoplasm appeared empty, probably representing inclusions of lipids, which had been extracted during the preparation procedure (Figs 5, 6 and 7)

Occasionally the cells had localized swellings (Fig 6) containing cytoplasm at the periphery and the presumed lipid material dominating the remaining part of the region The cell wall had the typical structure of gram negative bacteria (Fig 7), although the peptide layer between the cytoplasmic membrane and the outer undulated, three layered membrane was not particularly pronounced A flocculent material was observed external to the outer undulated layer, but whether it represents an additional layer of the cell wall or is derived from the substrate has not been determined

Motility

When cultures were examined *in situ* the gliding bacteria exhibited 3 types of movement, namely (1) a slow gliding movement of translation often punctuated by (2) a sudden rotation around one pole and (3) in the larger filaments flexion of the cell body also occurred In coverslip preparations gliding movement was only observed in localised areas

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Fig 4 A long slender rod with a blunted end is shown The cytoplasm is of medium electron density Strands of electron lucent material are seen outside the bacteria in close relation to the exterior of the cell wall A relatively large cytoplasmic protrusion is seen to the left No flagella are present Negatively stained with ammonium molybdate Magnification 60,800 × The bar represents 0 1 μm

Fig 5 Longitudinally sectioned micro organisms reveal that they are long slender rods Numerous partly empty spaces are seen in the cytoplasm probably representing inclusions of lipids, which have been extracted during the preparation procedure Magnification 10 600 × The bar represents 1μm

Fig 6 Section of an organism with a terminal swelling which contains cytoplasm at the periphery of the same appearance as that seen in the remaining part of the cell The presumed lipid material occupies a considerable part of the swelling Magnification 22 300 × The bar represents 0 5 μm

Fig 7 Higher magnification of the micro-organism showing a typical gram negative cell wall structure The peptide layer interposed between the cytoplasmic membrane and outer undulating three layered membrane is not very pronounced Flocculent material is seen in close association with the outer undulating membrane In the cytoplasm the nuclear zone is indistinct and a large part of the cytoplasm is occupied by empty spaces Magnification 95 000 × The bar represents 0 1 μm

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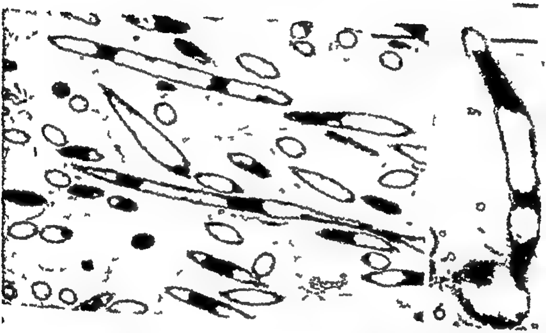

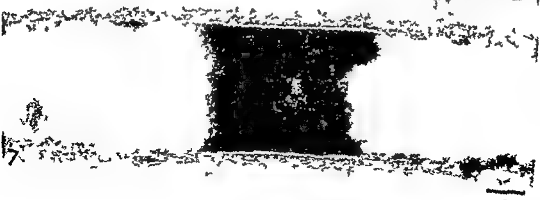

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TABLE 1 *Range of pH Values Recorded in Inoculated and Uninoculated Medium after 14 Days' Incubation*

	Gluc.	Lact	Mal	Suc	Xyl	Man	Sal
Uninoculated controls	6 5 6 7	6 6 6 7	6 5 6 6	6 5 6 6	6 4 6 5	6 5 6 7	6 5 6 6
Gliding bacteria (19 strains)	5 1 5 5	5 1 5 4	5 1 5 4	5 1 5 5	6 2 6 4	6 1 6 4	6 2 6 4

Biochemical Characteristics

All strains of gliding bacteria were catalase, oxidase, indole and H_2S negative In the fermentation tests the final pH values after 7 and 14 days' incubation did not differ appreciably and therefore only the values recorded on day 14 are shown in Table 1 Good growth of gliding bacteria was obtained in the presence of all the carbohydrates tested except salicin where growth was light and erratic

DISCUSSION

The similar colonial and cellular morphology biochemical characteristics and cell movements suggest that the 19 strains of anaerobic gram negative rods described form a homogenous group It would seem desirable to consider their relationship to other gliding gram negative rods previously isolated from the oral cavity

The three types of movement described, namely gliding, rotation around one pole and flexion of the cell body, have been observed in strains of *Fusocillus girans* (*Prevot* 1940) *Fusobacterium girans* (*MacDonald* 1953) and *Sphaerocytophaga sp* (*Gräf* 1961) Our observation that the strains were non-flagellated confirms the findings of *MacDonald* (1953) and *Gräf* (1961) The mechanism of gliding motility is still debatable (for a review see *Jahn* & *Bovee* 1965)

Free spheroid bodies were observed both in liquid and agar plaque medium and have been reported previously in cultures of *Fusocillus girans* (*Prevot* 1940) *Gräf* (1961) in a study of oral gliding rod shaped anaerobic bacteria, considered that the spheroid bodies were part of a characteristic life cycle Based on these features *Gräf* described a new genus *Sphaerocytophaga* (order Myxobacterales) Other workers (*Starr* & *Skerman* 1965 *Dworkin* 1966) considered the spheroids to be in fact spheroplasts and suggested that the organisms described by *Gräf* were anaerobic cytophagas In the present work the number of free spheroid bodies increased with age of the culture, further suggesting that the spheroids are in reality degenerating forms produced as a result of adverse environmental conditions

The strains described in the present study resemble *Fusocillus girans* (*Prevot* 1940) and Fusobacterium girans (*MacDonald* 1953) in being actively saccharolytic and indole and H_2S negative *MacDonald* (1953), however considered that there was inadequate justification for establishing the genus Fusocillus and proposed that it be named *Fusobacterium girans* The final pH values produced in glucose, lactose, maltose and sucrose by our strains are considerably lower than those reported for strains of *F girans* (*MacDonald* 1953) and *Sphaerocytophaga sp* (*Gräf* 1961) Although it would seem justifiable to consider all gliding species of fusobacterium as members of the genus Cytophaga as defined by *Stanier* (1942) *Lewin* (1969) considers that such a step would extend the bounds of this genus beyond convenient limits Until further biochemical and serological investigations are performed we have provisionally classified our strains as *Fusobacterium girans*

Although absent from the gingiva on day 0 the relative proportion of the gliding bac

teria increased in the early part of the experiment in all subjects It seems likely that these organisms are part of the normal oral flora and their temporary apparent increase during the first part of the study may possibly be due to a greater resistance to the action of chlorhexidine

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CHROMOSOME REPLICATION RELATED TO CELL DIVISION AND INCREASE IN CELL MASS IN SYNCHRONIZED CULTURES OF *NEISSERIA MENINGITIDIS*

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Synchronous growth of *Neisseria meningitidis* Strain M1 was obtained after release from prolonged chloramphenicol inhibition. Chromosome replication was examined by enumerating mutants induced with nitrosoguanidine in successive samples from the cultures and correlated with cell division as measured by colony forming units, and with growth as evaluated by increase in absorbancy. A transformable (genetically competent) variant of the test strain was compared with a non transformable (genetically incompetent) one. The two variants have been found to differ with regard to chromosome replication, presumably by *having different origin and direction of replication*. The experiments indicate that cell division in both variants starts when the same, definite region or locus on the chromosome is replicated. This region seems to be located close to the origin (or terminus) of replication postulated for the non transformable variant. The rate of increase in absorbancy is abruptly approximately doubled 5–7 minutes after the start of cell division, whereas it is constant during the division cycle. The sudden enhancement of the rate does not occur when the density of the population is higher than corresponding to about A = 0.600, although growth continues at higher densities. Some implications of these findings have been discussed.

Theoretical arguments as well as experimental data support the hypothesis that cell division in *Escherichia coli* and related bacteria must be dependent upon DNA replication (5, 6, 12, 13). The evidence indicates that completion of a round of replication is a necessary condition for cell division. But there seems to be disagreement as to whether the termination of a round of replication actually "triggers" a division.

In *E. coli* B/r cell division follows the completion of a round of replication by about 20 minutes at growth rates between 1 and 2 doublings per hour and by about ⅓ of a generation in cells growing more slowly (5, 13). As indicated by *Maaløe* (24) physiological division may occur at a time quite different from physical separation of the cells. However, experiments performed by *Clark* (6) with *E. coli* strain B/r indicate that even the physiological division is well displaced from the termination of a round of replication.

Chromosome replication in *Neisseria meningitidis* has been examined by two methods (16–18). First, relative frequencies of va

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rious genetic markers in the DNA from exponential and stationary growth phase were compared by use of a transformation system. These experiments were designed according to the model of *Sueoka* and *Yoshikawa* (30). Later, mutants induced by nitrosoguanidine during synchronous replication after release from prolonged chloramphenicol inhibition were enumerated by a modification of the method described by *Cerda Olmedo et al.* (4). The two series of experiments permitted the construction of replication maps which were in general agreement (16, 18, 19).

Chromosome replication was also compared in *N. meningitidis* variants which were genetically competent in DNA transformation, and variants which were genetically incompetent (16, 18). The change from competence to incompetence seemed to be followed by a change in the replication origin as well as in the direction of replication (18).

In the present study synchronous cultures of the *N. meningitidis* Strain M1 have been used to investigate the timing of the replication cycle in relation to cell division and rate of increase in absorbancy. A comparison between the competent and the incompetent variant has been carried out according to the following rationale.

If completion of a round of chromosome replication is sufficient condition of DNA synthesis for cell division, one would expect that the timing of cell division relative to the termination of the replication cycle is the same in the competent as in the incompetent variant. But if the replication of some chromosomal region is involved in the "triggering" of cell division, it should be possible to observe a difference in this timing because the genetic markers are replicated at different times in the replication cycles of the two variants (16, 18).

MATERIALS AND METHODS

Strains. The following nutritionally deficient mutants from the wild type strain M1 of *N. meningitidis* were used: M1–6 *his pro*, M1–8 *his arg* (18). Competence in transformation was controlled as previously described (17). Genetically competent variants were indicated by the symbol cp^{+} and genetically incompetent ones by cp^{-}.

Media. Blood agar plates and Heart Infusion Broth (HIB, Difco) agar plates were used as solid complete media. Fluid complete medium was Brain Heart Infusion Broth (BHI, Difco). The basal media were those previously described (15). Growth was followed by measuring the absorbancy as previously described (18, 19).

Synchronization of the growth. Chromosome alignment by means of chloramphenicol treatment and synchronization of the growth after release from the inhibition was performed according to the technique previously described (18, 19). A 20 ml amount of an exponentially growing culture of the test mutant was added to 100 ml of BHI. This culture was incubated on a shaker, usually for 3 hr at 37° C. Chloramphenicol was then added in a final concentration of 2.5 µg/ml, and the culture was incubated with shaking for another 2 hr at 37° C. Subsequently, the cells were harvested by centrifugation for 20 min at 2 500×g and resuspended in 4 ml saline. This suspension was used to inoculate 200 ml BHI prewarmed to the desired temperature. The culture was next incubated with regular stirring on a water bath. The growth was followed in samples taken at regular intervals, usually every 5 minutes. The sample was rapidly cooled by immersion in ice water for 15 sec. Absorbancy was measured and colony forming units determined by plate counts on blood agar plates. In some experiments the cultures were distributed in growth flasks provided with a side arm for the measurement of absorbancy.

Mutagenesis. Relative numbers of mutants during synchronous growth were measured in the following way (18, 19). Samples of 5 or 10 ml culture were taken every 5 min, cooled in ice water for 15 sec, centrifuged for 5 min at 2 500×g and resuspended in 1 ml saline. Mutagenesis with 3 µg/ml of nitrosoguanidine took place at 37° C on a water bath for 20 min. The suspensions were next diluted with 5 ml saline. The cells were recovered by centrifugation, washed with 5 ml saline and finally resuspended in 1 ml HIB.

Assays. All revertants to independence from nutritional requirements were tested by plating on basal media containing the required nutrients except the one being tested, and in addition 0.5 per cent HIB. Such an addition of limiting amounts of complete medium is necessary to obtain complete phenotypic expression of the mutations (16, 18). Selection of mutants to streptomycin resistance (Str r) was carried out by the technique previously described (23), usually allowing 5 hr for phenotypic expression before exposure to 100 µg streptomycin per ml. The inoculates during platings were chosen according to the mutation frequencies ob-

TABLE 1 *Sequential Mutagenesis in Synchronous Cultures of Competent Variants of* N meningitidis *Strain M1*

Expt No	Mutant analysed	Distance (replication units)			
		pro	*str*	*arg*	*his*
1	M1-8 *arg his cp*+		0	+0 13	+0 19
2	M1-8 *arg his cp*+		0	+0 12	+0 23
3	M1-6 *pro his cp*+	—0 19	0		+0 17
4	M1-6 *pro his cp*+	—0 26	0		+0 25

Synchronous growth at 32° C Samples taken every 5 min and mutagenized with nitrosoguanidine The average time between successive peaks of the same marker is used as a time unit and called the replication unit Values given are the average normalized distance from the Str r peak to the nearest peak of revertants in the same experiment

TABLE 2 *Sequential Mutagenesis in Synchronous Cultures of Incompetent Variants of* N meningitidis *Strain M1*

Expt No	Mutant analysed	Distance (replication units)			
		arg	*str*	*pro*	*his*
1	M1-8 *arg his cp*	—0 11	0		+0 74
2	M1 8 *arg his cp*	—0 12	0		
3	M1 6 *pro his cp*		0	+0 21	+0 89
4	M1-6 *pro his cp*		0	+0 18	+0 67

Experimental conditions and calculations as in Table 1

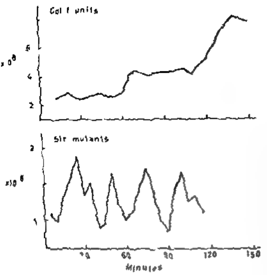
served in preliminary experiments so that the calculation should be based on counting no less than 100 colonies Surviving colony forming units were counted by the plating of appropriate dilutions on blood agar plates Graphs were constructed for the frequency of mutants obtained with the average of two plates of mutants and of two plates of viable counts

Chemicals N methyl N nitro N nitrosoguanidine (nitrosoguanidine) was obtained from Koch Light Laboratories Ltd Colnbrook Bucks, England It was dissolved in sterile, distilled water at 2 mg/ml and 1 ml portions were kept frozen until use Streptomycin sulfate was purchased from Glaxo Laboratories Ltd, Greenford England

RESULTS

The replication map Sequential mutagenesis in the auxotrophs M1-6-*his pro* and M1-8 *his arg* was first checked by mutagenesis with nitrosoguanidine (Table 1 and Table 2) The temperature was changed to 32° C during synchronous growth to slow down the rate of DNA replication (4 18) In each experiment three types of mutations were assayed and all experiments included the mutation to Str r and reversion in the *his* locus The average time difference between peaks of the same marker was used as a time unit and called the replication unit All markers were related to the Str r peaks in terms of replication units, calculated as the quotient of the time difference between the Str r peak and the peak corresponding to the marker in question over the replication unit

These results are in general agreement with those previously reported for the strain M1 (18 19) The sequence of the mutagenesis is as before and the distances between the peaks of mutants are nearly the same The experiments give further support to the hypothesis that the origin and direction of replication is changed upon the loss of the genetic determinant of competence (16 18 19)



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and treated with chloramphenicol as described in methods Synchronous growth was at 37° C Numbers refer to colony forming units per ml and to mutants per surviving colony forming unit after treatment with nitrosoguanidine

Relation between absorbancy and colony-forming units in synchronous cultures When plotted as in Fig 3 and Fig 4 the increase in absorbancy was linear, at least within the error of the method, during the first two division cycles But at certain times, an abrupt enhancement occurred in the rate of increase Apparently, the rate is changed 5-6 minutes after the start of cell division regardless of the competence When experiments with the cp variant were performed with more diluted suspensions, increase in absorbancy started after a lag of about 30 minutes after the release from chloramphenicol inhibition (Fig 4)

Each of the presumed linear parts of the growth curve was calculated from the experimental data by the method of least squares The lines thus constructed were used for the determination of rates, as well as for the time of the rate-change It was found that the rate was very nearly doubled each time it was changed, provided that the absorbancy was below approximately 0 600 At higher densities no rate change occurred, and the rate started to level off (Fig 3 and Fig 4) A vir-

Relation between cell division and the peaks of mutants in synchronous cultures The relation between the peaks of mutants and cell division was examined in experiments run at 37° C Fig 1 and Fig 2 show that the increase in the number of colony forming units occurs immediately after the second peak of Str r mutants in the cp^{+} variant whereas it appears just before the third peak in the *cp* one It is also seen that the first *Str r* peaks appear at widely different times after the release from chloramphenicol inhibition in the two variants These findings are in agreement with previous experiments in which synchronous growth took place at 32° C (19)

The experiments of Fig 1 and Fig 2 were repeated three times It appeared that the results could almost exactly be reproduced with the peaks of mutants respectively the increase in colony forming units appearing at the same points of time

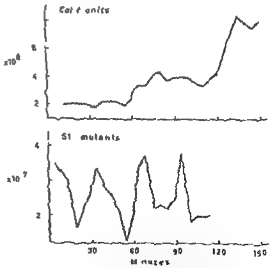

Fig 2 Resumption of growth and mutagenesis with nitrosoguanidine after release from chloramphenicol inhibition in a *cp* variant of *N meningitidis Strain M1* The auxotroph M1-8 *arg his cp* was grown and treated as in the experiment reported in Fig 1

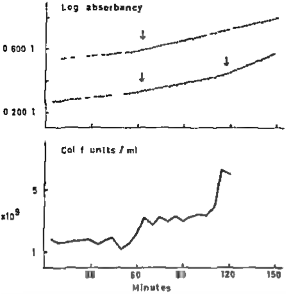

Fig 3 Resumption of growth after release from chloramphenicol inhibition in a *cp*⁺ variant of *N meningitidis* Strain M1 The auxotroph M1–8 *arg his cp*⁺ was grown and treated with chloramphenicol as described in methods Synchronous growth was at 37° C

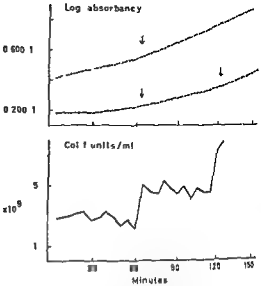

Fig 4 Resumption of growth after release from chloramphenicol inhibition in a *cp* variant of *N meningitidis* Strain M1 The auxotroph M1–8 *arg his cp* was grown and treated as in the experiment reported in Fig 3

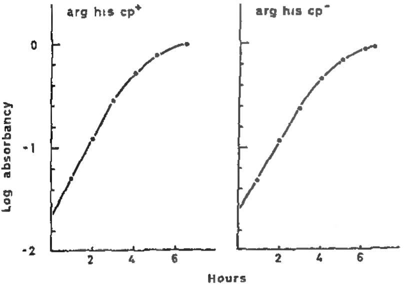

Fig 5 Batch cultures of the auxotroph M1–8 *arg his* Calculation of the generation time 8 *arg his cp* = 53 7 min 8 *arg his cp* = 62 3 min

tually linear curve is obtained in this region at least up to A = 0 800 by the use of an arithmetic plot

The growth of batch cultures of the test mutants is linear up to about A = 0 600 during exponential growth (Fig 5) This corresponds well with the density at which the rate increase in synchronous cultures is inhibited The average generation time was 54 minutes in four experiments with the *cp* variant and 58 minutes in four experiments with the *cp* one These generation times are

TABLE 3 *Relation between Increase in Colony Forming Units and Absorbancy, and the Peaks of Str r Mutants in Synchronized Cultures of N meningitidis Strain M1 Mutagenized with Nitrosoguanidine*

Test strains	Cell division or rate increase measured	Distance			
		Cell division to nearest preceding Str r peak		Rate increase to nearest preceding Str r peak	
		Minutes	Replication units	Minutes	Replication units
M1-8 *arg his cp*⁺	First	5 8	0 23	12 0	0 48
M1 6 *pro his cp*⁺	Second	11 3	0 45	16 0	0 64
*)	Ave	8 5	0 34	14 0	0 56
M1 8 *arg his cp*	First	19 8	0 66	26 5	0 88
M1 6 *pro his cp*	Second	16 5	0 55	22 5	0 75
*)	Ave	18 1	0 61	24 5	0 82

*) Two experiments performed with each of the strains Values given are the average of all four experiments Procedures were as described in the text to Fig 1 Start of cell division estimated from the number of colony forming units, and rate increase calculated from measurements of absorbancy as described in the text. The average distance between successive Str r peaks (the replication unit) was 25 min for the *cp*⁺ variants and 30 min for the *cp* ones

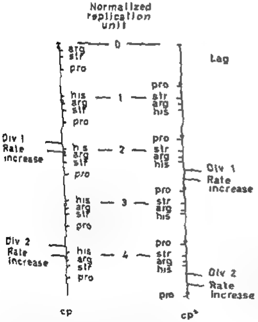

Fig 6 Cell division and sequential mutagenesis by nitrosoguanidine in synchronous cultures of *N meningitidis* Strain M1 The diagram has been drawn according to the data of Table 1 2 and 3 The origin of replication indicated by marker frequency analysis (16) has been indicated by the symbol *o*

not very different from those indicated by the estimation of colony-forming units in synchronous cultures

Timing of cell division in the chromosome replication cycle Table 3 shows the relation of the first two cell divisions to the second and the fourth Str r peak (Fig 1 and Fig 2) In these experiments viable counts were performed each 5 min, and the start of cell division was taken as the middle between the last time at which no change had occurred and the first time at which the number of colony-forming units was markedly increased In several experiments it was more difficult exactly to determine the start of the second division than the first one because the increase in colony forming units started more gradually The time at which rate-change took place was estimated from measurements of absorbancy by calculation as described above

Start of cell division such as measured follows average 8 5 min (0 34 replication units) after the preceding peak of Str r mutants in the *cp*⁺ variant, whereas it takes place average 18 1 min (0 61 replication units) after the preceding peak in the *cp*⁻ one The average

"lag" from the start of cell division to rate increase appears to be 5.4 min and 6.4 min in the cp^+ and the cp^- variants, respectively.

In Fig. 6 cell division and rate increase has been related to the (tentative) replication map by combining the data recorded in Table 1 and 2 with those of Table 3. The origin of replication has been (arbitrarily) mapped according to previous findings with marker frequency analysis (16).

DISCUSSION

It can be argued that these experiments are performed under very special physiological conditions. Several steps in the procedure may have changed the normal pattern of chromosome replication and cell division. Particularly, chloramphenicol treatment is known to have profound effects on the cell. Another problem is the fairly limited resolution of the techniques employed. The problems concerned with a more exact determination of the peaks of mutants have been discussed before (18). The difficulty in defining the start of cell division has been mentioned above, as well as by others (7). Since "cell division" is measured by the enumeration of colony-forming units, many of the features observed may be related to the diplococcal arrangement of *N. meningitidis*, and should not be directly compared with findings from rod shaped organisms like *E. coli*. These reservations must be kept in mind during the following interpretation.

The difference observed between the competent and the incompetent variant of the *N. meningitidis* Strain M1 is clear enough. The timing of cell division relative to the peaks of Str r mutants is entirely different. Both variants, however, have in common that the division time is very nearly twice the interval between two successive Str r peaks. The difference between the two variants seems to be a little more pronounced corresponding to the first cell division than to the second one (Table 3). This may be due to loss of synchrony (4, 18). The difficulty in determining exactly the start of the second cell division may be an indication of this, although the widths of the peaks of Str r mutants indicate that the loss is not very pronounced (4, 18).

The interval between two successive peaks of mutants has previously been used as "replication time" during mapping of various markers (18) and it was noted that the maps thus obtained corresponded well with those obtained by marker frequency analysis (16). Several models for chromosome replication in *E. coli* have postulated that the time required for a replication fork to traverse the chromosome cannot be less than 40 min at 37° C (7, 8, 25). Thus, the time between two successive peaks of mutants in *N. meningitidis* (approximately 25 and 30 min in the cp^+ and the cp^- variants, respectively) seems rather short compared to the replication time suggested for *E. coli*. A bidirectional replication like that suggested for *E. coli* (27) might possibly explain a short interval between two successive peaks. But like in *B. subtilis* (29, 30), there is so far no evidence to suggest that replication in *N. meningitidis* Strain M1 is not unidirectional (16).

When synchronous cultures of E. coli (TAU) are mutagenized with nitrosoguanidine (4) the time interval between successive peaks corresponds well with the generation time for exponential cultures in the same medium and temperature determined in independent experiments. In *N. meningitidis* the sum of two intervals corresponds well with the generation time in exponential cultures as well as with the division time in synchronized cultures.

When replication maps are constructed with the interval between two successive peaks of mutants as the unit ("replication unit") and interpreted according to the theories of *Cerda Olmedo et al.* (4, 18), the timing of cell division may be related to the origins of replication previously suggested for the cp^+ and the cp^- variants (16, 18). Like in *E. coli* cell division does not take place at a well defined time (7), but extends over a period of 15 to 20 min (Fig. 1, Fig. 2). Apparently, however, cell division in the cp^+ as well as in the cp^- variant starts at the time

when a locus or region near the *his* locus is replicated (Fig 6) This region is located close to the origin (or terminus) postulated for the chromosome of the *cp* variant, whereas it is distant from that suggested for the *cp*+ one (16, 18)

For these results, several models can be produced One is that two chromosomes are involved in the colony forming unit, and that alternation of synthesis of identical chromosomes takes place "Compartmentalization" or physiological division of the two units might for instance occur after the first replication whereas physical separation of the colony forming units could take place after the second one It has been suggested that this type of replication may take place in *E coli* when the bacteria grow at relatively slow rates although other evidence indicates that this is not so (2, 21, 22) But the findings from *N meningitidis* may also be explained by the existence of two replication forks during each division cycle, or two waves of mutants during each replication cycle Most of these models, however, remain too far removed from experimentation at the present stage to merit discussion

With some reservation absorbancy may be used as a measure for the cell mass (3 20, 25) The rate of increase in absorbancy, which is thus an expression for the total biosynthetic activity was abruptly enhanced 5–7 minutes after the start of cell division in the *cp*+ as well as in the *cp* variant The interpretation of these findings is not clear But it can be argued that the cell separation is a condition for the rate increase This hypothesis may also agree with the finding that the rate increase is inhibited when the density of the population is above approximately A = 0 600 Above this density mass accumulation seems to continue at a linear rate, and colony-forming units increase in number indicating that cell division still takes place

Mass increase has been measured during synchronous growth of several microorganisms (1 9 14 26 28) It seems that synchronization induced by growth to early stationary phase followed by resuspension leads to a pattern very much like that found in *N meningitidis* both in *E coli* and in *Proteus* (9) A selective procedure based on filtration also apparently results in a sudden enhancement in the rate of increase in mass (expressed by total nitrogen) following just after the beginning of cell division (26)

These observations bring to mind the contact inhibition studied in eucaryotic cells Contact inhibited cells exhibit a rate of formation of rRNA some two to four times lower than that shown by exponentially growing cells (11) It has been shown that duplication of the chromosome in *E coli* B/r has no effect on the synthesis of rRNA (10) This means that elements extrensic to the gene ("cytoplasmatic") must control the genes which code for rRNA and counteract any effect which gene replication might otherwise have One might thus speculate that the newly synthesized genes for rRNA in bacteria like *N meningitidis* are not expressed until contact between the daughter cells is interrupted

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ELECTRON MICROSCOPY OF ENDOTOXIC LIPOPOLYSACCHARIDE FROM *BACTEROIDES, FUSOBACTERIUM* AND *SPHAEROPHORUS*

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Lipopolysaccharide endotoxins (LPS) obtained by phenol water extraction of *Bacteroides melaninogenicus, B fragilis Fusobacterium* and *Sphaerophorus* and positively stained with uranyl acetate were studied in the electron microscope The LPS particles had characteristic morphological features comparable to those of LPS from other anaerobic as well as aerobic Gram negative bacteria LPS particles from *B melaninogenicus* and *Fusobacterium* mostly appeared as discs delimited by a single or a triple layered surface structure The preparations also contained rod like particles exhibiting a similar trilaminar structure LPS particles from *B fragilis* and *Sphaerophorus* appeared mainly as rods In addition LPS prepared from *Sphaerophorus* contained long ribbon like structures Different ways of purification or differences in chemical structure of the basal polysaccharide core of the LPS seemed to have no discernible effects on the morphology of the extracted particles Experiments with ferritin labelled antibodies to *B melaninogenicus* LPS suggested a superficial location of LPS in the bacterial cells but no further details

A variety of particles of different morphology has been observed by electron microscopy of lipopolysaccharides (LPS) isolated from aerobic bacilli (15 18, 23, 24, 25) The particles described include straight or curved rod like trilaminar structures discs or spheroids and long ribbons frequently with branching LPS from the anaerobic coccus *Veillonella* has the appearance of flat or twisted discs (2) The observed structures have been interpreted as micellar aggregates of linear lipopolysaccharide sub units (18, 20) or as ordered structures of phospholipid like bimolecular leaflets (21, 23)

The present report is concerned with the electron microscopic appearance of LPS extracted by 45 per cent aqueous phenol from the anaerobic bacilli *Bacteroides melaninogenicus, B fragilis Sphaerophorus necrophorus* and *Fusobacterium* The chemical composition, and some biological properties of the isolated LPS preparations have been reported (4, 5 6 7, 8 10 13, 14) Most of the preparations showed an overall quantitative chemical composition roughly similar to that reported for LPS of *Enterobacteriaceae* and other Gram negative aerobic bacteria How-

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ever, LPS isolated from *B melaninogenicus* and *B fragilis* differed markedly from LPS from most other bacteria including *Fusobacterium* and *Sphaerophorus* in their sugar composition, in that they lacked heptose and 2-keto-3-deoxyoxtonate (KDO) When present, heptose has been found to be the basal sugar of the polysaccharide core (16), while KDO serves as a link between this core and the lipid moiety of the macromolecular LPS complex (19)

The ultrastructure of LPS lacking KDO and heptose in the basal polysaccharide core has not previously been reported The purpose of this study was to compare the ultrastructure of *Bacteroides* LPS with that of LPS of *S necrophorus* and *Fusobacterium,* and with the different morphological structures of bacterial LPS observed by others We have also investigated whether LPS preparations purified in different ways differed in their electron microscopic appearance, as well as the location in the bacterial cell of *B melaninogenicus* LPS Parts of the study have been preliminarily reported (22)

MATERIALS AND METHODS

Bacterial strains Isolation and cultivation of *B melaninogenicus* B10 and *Fusobacterium* F1 have been described previously (4, 12) *B fragilis* NCTC 9343 and *S necrophorus* N167 Fievez were furnished by Dr *Ella Barnes,* Food Research Institute Norwich England Cultivation was performed as reported earlier (7 10)

Isolation and purification of LPS have been described (4, 7, 13) In short acetone dried whole cells of *B melaninogenicus* B10 *B fragilis* NCTC 9343 and *S necrophorus* N167 or crushed, defatted cells of *Fusobacterium* F1 were extracted at room temperature with 45 per cent aqueous phenol and the water phase fractionated by ultracentrifugation at 100 000×g for 1 hr The washed pellets ob [illegible] are referred to as

[illegible]

tions obtained in this way are designated LPS B10 sup and LPS N167 sup

Immunoferritin technique 0 2 ml of rabbit antiserum against LPS B10 sed (5) was mixed with 2 ml of a saline suspension of 10^9 microbes per ml of washed cells of *B melaninogenicus* B10 The mixtures were allowed to stand at room temperature for 30 min Following three washings in saline the bacterial cells were suspended in 2 ml of saline and mixed with 0 05 ml of a ferritin IgG conjugate of goat anti rabbit gamma globulin (Ferritin conjugate Kit Lot No 4117 Cappel Laboratories, Inc , Downington Pa) Incubation was carried out at room temperature for 30 min and the treated cells washed as described Control experiments in which normal rabbit serum was substituted for antiserum LPS B10 sed were included

Electron microscopy Lyophilized LPS was suspended by sonication for up to 2 min at concentrations of 0 2 or 0 5 mg per ml positively stained with uranyl acetate at pH 4 5, and prepared for electron microscopy as described by *Shands et al* (23) The ferritin labelled bacteria were fixed in 1 per cent osmium tetroxide in Veronal acetate buffer (11) transferred to agar and embedded in epoxy resin Thin sections for electron microscopy were cut with glass knives, collected on carbon coated formvar membranes and examined in the electron microscope without additional contrasting The electron microscope (Philips EM 300) was operated at 80 kV and micrographs were obtained at plate magnifications up to *60 000 diameters*

RESULTS

LPS melaninogenicus B10

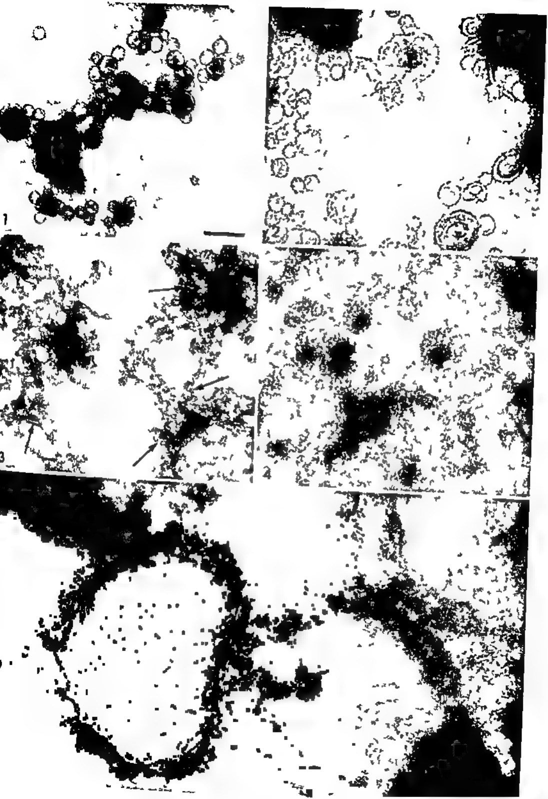
Lyophilized LPS purified by sedimentation in the ultracentrifuge LPS-B10 sed , appeared

Figs 1 and 2 Lyophilized lipopolysaccharide endotoxin (LPS) extracted from *Bacteroides melaninogenicus* with phenol water and purified by sedimentation in the *ultracentrifuge* Electron micrograph magnification × *100 000* Horizontal bar in Fig 1 represents 1000 Angstrom Note *that all figures are reproduced at the same magnification*

Fig 3 Lyophilized LPS from *B melaninogenicus* isolated from the supernatant *fluid* after ultracentrifugation A few rod like and disc like structures can be identified (arrows) × 100 000

Fig 4 Undialysed water phase after extraction of *B melaninogenicus* B10 cells with 45 per cent phenol Note LPS particles × 100 000

Fig 5 Location of LPS in sectioned *cells of B melaninogenicus Aggregations of ferritin* particles indicate that LPS is located at the outer layer of the cell wall × *100 000*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as globules or disc like particles varying in diameter from 250 to 1200 Å (Fig 1) Most of the particles were in the range 250–400 Å The particles appeared to be delimited by a dense line, or by a triple layered surface The latter structure consisted of two electron dense lines, about 20 Å in width, separated by an electron lucent space, about 50 Å in width The inner electron dense line was sometimes less distinct than the outer one, and the tri laminar delimitation did not always form a complete circle (Fig 1)

The LPS B10 sed preparations also contained a few rod like particles measuring 90 Å in width and 250–400 Å in length (Fig 2) The rods showed a double tracked structure with dimensions similar to the trilaminar structure delimiting the disc like particles The two parallel electron dense lines sometimes seemed to converge, but did not meet at the ends of each particle

The LPS isolated from the supernatant fluid after ultracentrifugation was seen in the electron microscope as a rather dense aggregated material in which definite structures were hard to discern Occasionally however, rod like and disc like structures similar to the LPS B10 sed preparations could be identified (Fig 3)

In one of the experiments the purification of LPS B10 sed was followed through all preparatory steps by electron microscopy In this experiment suspension of LPS by sonication was omitted The undialyzed water phase after extraction with 45 per cent phenol contained disc like particles similar to those observed in lyophilized LPS B10 sed preparations (Fig 4) although less clearly resolved Micrographs of the pellet obtained by ultra centrifugation also showed a picture closely resembling that of lyophilized LPS B10 sed Similarly, there was no difference in the electron microscopic appearance of the super natant fluid following ultracentrifugation and the lyophilized LPS B10 sup

The location of LPS in the bacterial cells was studied by means of the indirect ferritin conjugate antibody technique The most conspicuous finding in sections of *B melaninogenicus* B10 cells treated with rabbit anti serum against LPS B10 sed and ferritin labelled antibodies to rabbit gamma globulin was the presence of electron dense aggregations of ferritin particles surrounding the bacteria immediately peripheral to the outer triple layered cell membrane (Fig 5) Ferritin was not found in the cytoplasm or inside the cytoplasmic membrane of seemingly intact or disintegrating cells

LPS B fragilis NCTC 9343

Lyophilized LPS 9343 appeared in the electron microscope as a packed mass of poorly resolved rod like particles and possibly spherical structures (Fig 6) The size of the rods was within the range described for LPS B10 sed No trilaminar membrane like structures were seen

LPS S necrophorus N167

The appearance of LPS N167 purified by sedimentation in the ultracentrifuge was that of weakly stained coalescing ribbons distinctly different from the structures observed in the other LPS preparations (Fig 7) The ribbons were 90–130 Å in width Part of the ribbon like material had a trilaminar structure Some short rods and some relatively electron dense spherical particles with a diameter of 400–1000 Å possibly representing aggregated ribbons were also present

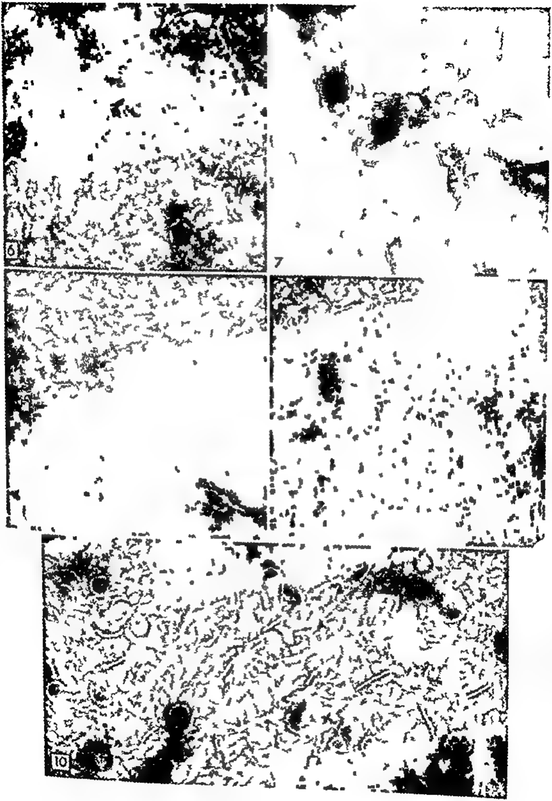
LPS purified by gel filtration and ion exchange chromatography consisted of granular material without definite structure and of 'trilaminar' rods similar to those previously described (Fig 8)

Fig 6 Lyophilized LPS from *B fragilis* × 100 000

Fig 7 Lyophilized LPS from *S necrophorus* purified by sedimentation in the ultracentrifuge × 100 000

Fig 8 Lyophilized LPS from *S necrophorus* purified by gel filtration and ion exchange chromatography × 100 000

Figs 9 and 10 Lyophilized LPS from *Fusobacterium* × 100 000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LPS Fusobacterium *F1*

Some preparations of LPS-F1 were dominated by closely packed straight and curved rods (Fig 9) All preparations contained large numbers of disc-like particles, 250–1200 Å in diameter The particles had a single or a trilaminar surface membrane (Fig 10)

DISCUSSION

The electron microscopic study of phenol-water extracted LPS from the Gram negative anaerobic microorganisms *B melaninogenicus, B fragilis, S necrophorus* and *Fusobacterium* revealed a variety of particles differing in shape and size The exact three dimensional structure of these particles, however, remains to some extent uncertain The particles described as discs and spheres may represent flat or twisted discs with or without indented centres, or they may be globules The rod-like particles may be discs standing on edge or cylindrical bodies The ribbons may be interpreted as sheets or tubules

The study did not disclose unknown particulate structures The morphological forms observed in electron micrographs of LPS isolated by sedimentation in the ultracentrifuge, for instance, have been found in preparations of LPS prepared in different ways from strains of *Escherichia coli* and *Salmonella typhimurium* (1, 15, 23) The same is true for the rod like particles in LPS N167 sup and the particulate structures observed in preparations of supernatant LPS B10 Within the limitations of the techniques used, the particles seen in preparations of the KDO- and heptose less LPS from *B melaninogenicus* and *B fragilis* NCTC 9343 were judged to be morphologically similar to supernatant LPS from *S necrophorus* N167 and to *Fusobacterium* LPS Moreover, the disc like structures with single- or triple layered delimitations conspicuous in LPS of the oral strains *B melaninogenicus B10* and *Fusobacterium* F1 have also been observed in phenol-water extracted LPS preparations from oral *Veillonella* (2), which contain heptose and relatively large amounts of KDO (8) Differences in chemical structure of the basal polysaccharide core in LPS therefore were not reflected in the morphology of the extracted LPS particles

LPS prepared from strain B10 by fractionation of the supernatant fluid following ultracentrifugation was not distinctly different from the pellet LPS The same applies to the rod-like structures observed in LPS sed and LPS sup prepared from strain N167 Purification of LPS by means of gel filtration and ion exchange chromatography, which is an alternative method for obtaining a glycogen free, serologically and biologically active LPS thus yields LPS fractions with a morphology similar to that of LPS purified by sedimentation by ultracentrifugation It is not clear whether the rods seen in preparations of LPS from strain N167 represent shorter segments of broken-up ribbon like structures or whether these particles are a separate entity

The morphology of extracted LPS has been found to be subject to alterations during purification, including lyophilization and dispersion by sonication (23) In this study, however, the structure found in the separated water phase after phenol water extraction of acetone-dried B10 bacteria were the same as those observed in lyophilized LPS B10 sed and LPS-B10 sup preparations, regardless of treatment with ultrasonic before preparation for electron microscopy

According to expectation, the experiments with ferritin labelled antibodies suggested a morphological association of *B melaninogenicus* LPS with the outer parts of the bacterial cell wall However the electron micrographs were inconclusive with respect to the more detailed location of LPS within the cell wall The question whether the LPS particles originate from the outer triple layered cell wall membrane (17) or from other superfical cell wall structures (3) remains *unsettled*

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INTRAVASCULAR CLEARANCE OF *ENDOTOXIN* IN WARFARIN—TREATED RABBITS

The Influence of Immune Serum on Clearance and Distribution

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The intravascular clearance of intravenously administered endotoxin prior to and after administration of immune serum has been compared in untreated rabbits and in rabbits pretreated with warfarin sodium for 4 and 66 days respectively Firstly the present observations show that the phagocytic rate of endotoxin without immune serum is significantly higher in rabbits exposed to short term warfarin pretreatment than in those exposed to long term pretreatment and in untreated rabbits The long term warfarin treatment is supposed to depress the function of the reticuloendothelial system particularly in the liver Secondly the phagocytic rate of endotoxin increases significantly following the administration of immune serum which almost completely abolishes the difference between the phagocytic rates of the groups prior to administration Administration of immune serum seems to decrease the uptake of endotoxin in some organs or provoke an immediate redistribution of the endotoxin between the organs The warfarin pretreatment influences the relative weight of the liver and the duration of the warfarin pretreatment seems to influence the organ localization of endotoxin

Intravenously administered endotoxin disturbs the dynamic equilibrium of the coagulation system, a decrease in the function of the reticuloendothelial system (R E S) (12) and changes in several other mechanisms taking place concurrently (18) In a previous study (12) a short pretreatment of rabbits with warfarin sodium gave an increased intravascular clearance and somewhat higher organ localization of intravenously administered endotoxin In that study (12) the rabbits were nontolerant and no specific antibodies against the injected endotoxin influenced the clearance and the distribution in organs

In the presence of specific antibodies in blood there is an increased clearance rate and a changed organ uptake of endotoxin in non warfarin treated rabbits (4 14) The main intention of the present work has been to examine how pretreatment with warfarin sodium influences the clearance and distribution of endotoxin in rabbits after administration of immune serum

Adlercreutz et al (1) found that the duration of the warfarin sodium pretreatment had a decisive effect on the blood clearance of gold in man The secondary intention has therefore been to ascertain whether the duration of warfarin sodium pretreatment affects the clearance of endotoxin—*both* prior to and after administration of immune serum

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MATERIALS AND METHODS

Animals Thirty ' White land rabbits caged, fed and chosen as described in an earlier paper (9), were grouped in three groups A, B and C, each comprising 5 male and 5 female rabbits. The mean weights of the rabbits were 4 3 kg (Variation 2 5–5 2), 4 3 (Variation 3 5–5 4), and 4 0 (Variation 3 3–5 2), respectively There was no significant difference between the weights of the groups The average age was 7½ (7½–7½), 7¾ (7½–8) and 7¼ (6½–7½) months

Anticoagulant treatment with warfarin sodium (warfarin) and Thrombotest determination of the coagulation activity in per cent (TT%), were carried out as described earlier (10) Groups A and B of the rabbits were chosen at random for anticoagulant treatment.

Endotoxin Bacto Lipopolysaccharide B (L P) from *S typhi 0901* (Difco) (control No 511771), stored at 4° C was used

⁵¹Cr labelling of endotoxin Chromium 51 was delivered as chromic chloride in isotonic sterilized solution with pH 4 0 and specific activity 142 2 mC/mgCr and 82 2, respectively (The Radiochemical Centre, Amersham/Institutt for Atomenergi Kjeller, Norway) The labelling was performed as earlier described (12) In the dialyzed mixtures 1 mg endotoxin bound 1 95 and 1 90 mcg Cr respectively The rabbits were given 0 15 mg endotoxin/kg body weight (b w), by injection in the left ear vein in 30 seconds

Immune serum Rabbits were injected intravenously with formalin—killed *S typhi 0901 H'* bacteria Pooled immune serum from the rabbits having a bacterial 0 agglutination titre of 1 2560 was stored at —20° C in small portions, and thawed and heated to 38° C before injection in the left ear vein The rabbits were given 1 0 ml serum/kg b w

Bacterial agglutination test was performed as previously described (11)

The volume of packed red cells (V P C) and the *erythrocyte sedimentation rate* (E S R) were determined according to the technique described by *Wintrobe* (23)

Blood samples 0 5 ml volumes were obtained from the right marginal ear vein, larger volumes being collected after the rabbits were killed The blood samples were handled as described earlier (12)

Autopsy and organ specimens Twenty min after endotoxin injection the rabbits were killed and bled out After gross examination, the lungs, heart liver spleen and kidneys were removed and weighed The relative weighs of the various organs i e organ weight/animal weight was calculated. Specimens were taken from each organ as previously described (12)

Radioassay was carried out with a scintillation counter 6006 IDL equipped with a well-type scintillator (NaI(TA) crystal 13/4" × 2) The samples, in liquid form and in equal volumes, 3 0 ml, were placed in flat bottomed tubes fitted to the well

Vascular clearance (disappearance) curve was drawn and resolved in two components The first represented the last 5 endotoxin concentrations prior to, and the second the 5 concentrations subsequent to, injection of immune serum (12)

The phagocytic index, k, and the clearance half time, T/2, for the two components were calculated (12)

Statistics The arithmetic mean was used, and the standard error (S E) was calculated by common statistical methods Analysis of variance (two-way classification) and regression analysis using the method of the least squares were performed according to *Sverdrup* (21) The significance of the difference between two means was tested by the t test A 5 per cent (0 05) level of significance was used throughout

EXPERIMENTS AND RESULTS

A comparison was made between the three groups of rabbits (A, B and C) with regard to the intravascular clearance and to the organ distribution of intravenously administered endotoxin

The rabbits of all groups were injected with endotoxin, and 9½ min after injection all were intravenously injected with immune serum The endotoxin clearance was recorded prior to and after the immune serum injection The distribution of endotoxin in the organs was determined at autopsy 20 min after endotoxin injection The examination of each group was performed over a period of 2–3 days, groups C and A being examined successively, and group B 18 days later

The O-antibody titre against *S typhi 0901* was in all cases below 1 16 prior to endotoxin injection and the rabbits were recorded as nontolerant

The mean V P C of the three groups A B and C was 41 0 (range 38–44), 44 5 (range 38–48) and 44 1 (range 41–47) ml/100, respectively and the mean E S R. 1 7 (range 1–2) 1 6 (range 1–3) and 1 3 (range 1–2) mm/hr respectively

The rabbits of group A were chosen at random for "long-term" warfarin pretreat

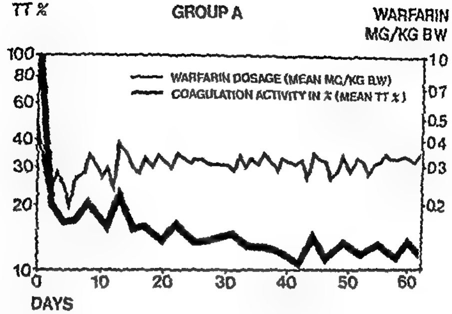

Fig 1 The mean coagulation activity in % (mean TT%) and the mean warfarin dosage (mean mg warfarin/kg b w) for the long term warfarin treated rabbits (group A) *during* the 9 weeks prior to endotoxin injection

TABLE 1 *The Mean and S E of Endotoxin Content in Peripheral Blood for Warfarin Treated (Group A and B) and Untreated (Group C) Rabbits at 1 Min Intervals in the Periods 3½-8½ and 10½ 17½ Min after Endotoxin Injection Immune Serum was Injected 9½ Min after Endotoxin*

Minutes after inject	Endotoxin (mcg ³/ml)					
	A		B		C	
	Mean	S E	Mean	S E	Mean	S E
3½	1429	102	1191	81	1300	67
4½	1247	94	1034	77	1169	68
5½	1143	81	939	70	1104	68
6½	1083	89	869	68	1033	79
7½	1027	93	818	71	992	74
8½	999	94	757	66	935	74
9½ (Immune Serum)						
10½	916	112	633	60	825	90
11½	712	85	468	46	630	65
12½	565	74	364	40	499	46
13½	455	58	288	32	401	31
14½	381	46	239	28	321	26
15½	327	40	203	23	286	18
16½	285	38	186	23	213	19
17½	257	34	164	20	226	20

ment for the last 66 days prior to endotoxin injection (Fig 1) One male rabbit died probably due to the anticoagulant treatment The mean TT% of the others was 13 9 for the last 60 days and 9 8 (range 4 5-14 0) on the day of endotoxin injection The rabbits of group B were given short term warfarin treatment for the last 4 days prior to endo-

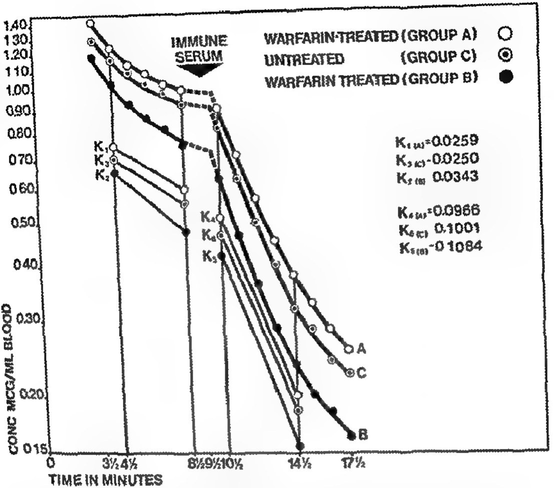

Fig 2 The clearance curve for intravenously administered endotoxin in "long term" (group A) and in "short term" (group C) warfarin treated rabbits and in untreated rabbits for the period 3½–17½ min after injection. Immune serum was administered 9½ min after endotoxin injection. The straight lines represent the linearized clearance curves for the periods 4½–8½ and 10½–14½ min respectively. The absolute values of the regression coefficients for the lines K_1–K_6 correspond to the phagocytic indices.

toxin injection. The mean TT% was 9.4 (range 6.0–12.5) on the day of injection.

The Intravascular Endotoxin Clearance

The concentration of endotoxin in whole blood was determined in each rabbit at 1 min intervals in the periods 3½–8½ min and 10½–17½ min after the endotoxin injection, immune serum being administrated at 9½ min. For each group the mean concentration and S.E. at each interval is given in Table 1. Throughout the whole period of examination (3½–17½ min.) the individual variations seem to be a little higher for the rabbits of group A than for those of group B and C, which were fairly similar.

For all intervals both prior to and after administration of immune serum the mean concentrations are highest in the "long term" warfarin treated rabbits (group A) (Table 1). Prior to immune serum administration variance analysis shows a significant difference between the concentrations of group A and B and between those of group B and C ($p < 0.01$) but no difference between those of group A and C. After the immune serum

TABLE 2 *The Clearance of Endotoxin from Blood in Warfarin-Treated (Group A and B) and Untreated (Group C) Rabbits Expressed by the Mean Phagocytic Index (K), and the Half Clearance Time (T/2) for the Period 4½–8 Min and the Period 10½–14½ Min after Endotoxin Injection i.e. prior to and after Immune Serum Injection*

Group	Period 4½–8½ Min			Period 10½–14½ Min		
	K × 10^2 Mean	S.E.	T/2 (min) Mean	K × 10^2 Mean	S.E.	T/2 (min) Mean
A	2.59	0.37	11.62	9.66	0.53	3.12
B	3.43	0.23	8.78	10.84	0.71	2.78
C	2.50	0.28	12.04	10.01	0.81	3.00

administration, there is a significant difference between the concentrations of each of the groups, the difference between those of group A and C being barely significant ($0.01 < p < 0.05$)

The mean concentrations of each group are visualized by the clearance curves in Fig 2 For all groups the curves become abruptly steeper after immune serum administration, demonstrating a rapid fall of endotoxin concentration

An approximation was made to linearize the equations of two components of each clearance curve (Fig 2) The two components represented the last 5 endotoxin concentrations prior to (4½–8½ min) and the first 5 concentrations after (10½–14½ min) immune serum administration

Based on this approximation the endotoxin clearance rate as expressed by the phagocytic index, K, and the half-time, T/2, was calculated The results are given in Table 2 The

TABLE 3 *The Mean and S.E. of the Absolute and the Relative Weights of Various Organs of Warfarin Treated (Group A and B) and Untreated (Group C) Rabbits respectively at Autopsy i.e. 20 Min after Endotoxin Injection*

Organ	Group	Absolute weight (g) Mean	S.E.	Relative weight (g/kg b.w.) Mean	S.E.
Liver	A	108.06	7.93	25.25	1.36
	B	101.71	6.74	23.61	0.70
	C	122.44	9.53	30.79	1.31
Spleen	A	0.92	0.12	0.23	0.04
	B	1.23	0.11	0.29	0.03
	C	1.28	0.13	0.32	0.03
Kidneys	A	22.49	0.98	5.39	0.45
	B	21.75	1.15	5.07	0.11
	C	21.03	1.12	5.32	0.12
Lungs	A	14.36	0.42	3.45	0.28
	B	14.00	0.95	3.28	0.18
	C	15.52	1.08	3.93	0.18
Heart	A	8.83	0.34	2.10	0.11
	B	9.13	0.41	2.15	0.09
	C	9.48	0.35	2.42	0.07

TABLE 4 *The Mean and S.E. of the Endotoxin (L.P.) Content in Various Organs of Warfarin Treated (Groups A and B) and Untreated (Group C) Rabbits, respectively, at Autopsy t = 20 min after L.P. Injection. The L.P. Content is Presented as Total Amount per Organ, as the Amount per Gram Organ Weight, and as % of the Injected L.P. Dose*

Organ	Group	Total L.P. mcg		L.P./g organ mcg/g		L.P./inj. L.P. %	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Liver	A	416.0	23.0	3.92	0.21	64.70	1.46
	B	433.7	23.4	4.31	0.12	67.33	1.20
	C	408.0	20.3	3.41	0.13	68.64	1.54
Spleen	A	6.0	1.2	6.56	0.89	0.90	0.15
	B	9.8	1.2	7.90	0.54	1.55	0.19
	C	10.6	1.9	8.13	1.14	1.77	0.32
Kidneys	A	2.1	0.3	0.10	0.01	0.32	0.03
	B	2.0	0.2	0.09	0.01	0.31	0.03
	C	2.0	0.2	0.10	0.01	0.34	0.03
Lungs	A	42.7	6.2	2.94	0.40	6.34	0.75
	B	59.5	5.9	4.35	0.48	9.33	0.90
	C	42.3	6.7	2.68	0.34	6.90	0.89
Heart	A	0.4	0.1	0.04	0.01	0.05	0.01
	B	0.3	0.1	0.04	0.01	0.05	0.01
	C	0.05	0.01	0.05	0.01	0.08	0.01
Liver Spleen Lungs	A	464.6	28.5			71.94	1.41
	B	503.0	24.6			78.21	1.26
	C	460.8	25.4			77.21	1.23
All organs	A	467.1	28.8			72.32	1.43
	B	505.3	24.7			78.58	1.28
	C	463.3	24.7			77.63	1.28

individual clearance rates vary relatively little, being of about the same magnitude in all groups. For the period prior to immune serum administration (4½–8½ min.), the phagocytic index of group B is significantly higher than that of group A and group C. The phagocytic index of group C is approximately that of group A. For the period after immune serum administration (10½–14½ min.), the "long term" warfarin-treated rabbits (group A) have the lowest and the "short term" warfarin treated (group B) the highest phagocytic index, but the difference between the phagocytic indices of the groups is not significant.

In each of the groups the phagocytic index is significantly higher following immune serum administration than before.

The findings thus show that in the period prior to immune serum administration intravenously injected endotoxin disappeared from circulation at a significantly higher rate in "short term" warfarin-treated than in "long term" warfarin treated and in untreated rabbits. Moreover, the immune serum provokes a significantly higher clearance rate in all groups than was obtained before the presence of immune serum, and seems to abolish the difference between the clearance rates of the groups.

The Distribution of Endotoxin in Organs

The mean and S.E. of the absolute and relative weights of the various organs in the three groups (A, B and C), are given in Table 3. The individual corresponding organ

TABLE 5 *The Statistical Difference between the Three Groups (A, B and C) on Comparison of Organ Weights and Content of Endotoxin (L P) in Various Organs of the Groups*

Groups compared	Organ	Weight: Absolute weight Signif	Weight: Absolute weight N signif	Weight: Relative weight Signif	Weight: Relative weight N signif	Endotoxin (L P) content: Total L P Signif	Endotoxin (L P) content: Total L P N signif	Endotoxin (L P) content: L P /g org Signif	Endotoxin (L P) content: L P /g org N signif	Endotoxin (L P) content: Percentage inject L P Signif	Endotoxin (L P) content: Percentage inject L P N signif
A/B	Liver		–		–		–		–		–
A/C			–	**			–		–		–
B/C			–	**			–	**			–
A/B	Spleen		–		–	*			–	*	
A/C			–		–		–		–	*	
B/C			–		–		–		–		–
A/B	Kidneys		–		–		–		–		–
A/C			–		–		–		–		–
B/C			–		–		–		–		–
A/B	Lungs		–		–		–	*		*	
A/C			–		–		–		–		–
B/C			–	*			–	*			–
A/B	Heart		–		–		–		–		–
A/C			–	*			–		–		–
B/C			–	*			–		–		–
A/B	All organs						–			**	
A/C							–			**	
B/C							–				–

– $p > 0.05$
* $0.01 < p < 0.05$
** $p < 0.01$

weights, especially the relative weights, vary comparatively little The individual variations are for most of the organs about the same in all groups No significant difference was found between the three groups for any of the corresponding absolute organ weights Nor was there any difference between the two warfarin-treated groups (A and B) with regard to corresponding relative organ weights There was however, a significant difference between corresponding relative weights of some organs when the two warfarin-treated groups were compared with the untreated group The statistical results are summarized in Table 5

Distribution of endotoxin among liver, spleen, kidneys, lungs and heart at 20 min after injection is shown in Table 4 as the content per organ as amount per g organ weight (concentration), and as per cent of the injected endotoxin dose The individual variation within each group is small, especially with regard to content in the liver For the same organ the mean values obtained by the three methods have about the same relative variation, but for most organs the variation of the percentage of injected dose is the lowest one

It should be noted (Table 4) that in all groups more than 70 per cent of the injected dose is located in the liver, lungs and spleen and that the major part is in the liver Less

than 0.5 per cent is located in the kidneys and only traces are found in the heart. The concentration, i.e. endotoxin per g organ, is in all groups highest in the spleen.

When two and two groups are compared as shown in Table 5, the t test shows a significantly higher percentage of injected dose in the five organs in groups B and C than in group A and a little but not significantly higher percentage in group B than in group C.

The results are more complex (Table 5) when the contents in the various organs from the three groups are compared. It should however, be noted that group B has a significantly higher percentage of injected endotoxin both in the spleen and in the lungs and a significant higher endotoxin concentration in the lungs than group A. Group B has also a significantly higher endotoxin concentration than group C both in the liver ($p < 0.001$) and in the lungs.

Endotoxin in Peripheral Blood

The absolute and relative amounts of endotoxin located to blood cells and the plasma phase were examined separately in blood collected at autopsy.

The percentage of endotoxin in whole blood located to the plasma phase was calculated with regard to the individual V.P.C. The *mean* value in per cent and S.E. for each group is presented in Table 6. The variation between individual animals is relatively small in all three groups. The whole blood concentration is highest in the "long term" warfarin treated and lowest in the "short term" warfarin treated rabbits consistent with the clearance results. The difference, however, is not significant. In all groups more than 92 per cent of circulating endotoxin is located to the plasma phase, and there is no significant difference between the groups with regard to the distribution of endotoxin between formed elements and the plasma phase.

It is noteworthy that the present absolute amounts of endotoxin in whole blood are obtained by another method than those of the clearance study, and a direct comparison is therefore of little value.

TABLE 6. *Endotoxin Content 20 Min. After Injection in Whole Blood and the Percentage Located to the Plasma Phase of the Blood in "Long Term" (Group A) and "Short Term" (Group B) Warfarin Treated and in Untreated (Group C) Rabbits*

Group	Endotoxin Content			
	Whole blood (mcg $^{-3}$/ml)		Plasma phase (%)	
	Mean	S.E.	Mean	S.E.
A	195	27	94.54	1.14
B	145	15	9[illegible].14	1.29
C	166	14	95.46	0.77

DISCUSSION

The presented results support the conclusion drawn in a previous paper (12) that intravenously administrated endotoxin is cleared from circulation at a higher rate in "short term" warfarin treated than in untreated rabbits. The results (Table 1 and 2) are reflected in the first part of the clearance curve (Fig. 2). The rabbits are regarded as nontolerant for this part of the clearance study. The present clearance rate in untreated rabbits is of the same magnitude as in the previous work (12) and is the same as that obtained by others in nontolerant rabbits (13–20). However rabbits pretreated with warfarin for 9 weeks have a significantly lower clearance rate than "short term" warfarin treated rabbits and seem also to have a somewhat lower rate than untreated rabbits.

The coagulability of blood as determined by the Thrombotest is the same in the two warfarin treated groups. If phagocytic rate were dependent on coagulability one would expect an equal rate in the two warfarin treated groups and that the rate in both groups would be higher than in the untreated group (12). The present findings however show that pretreatment with warfarin over a long period depresses the capacity of the R.E.S. to remove endotoxin from circulation. This is according to the observation of

Adlercreutz et al (1) that phagocytosis of gold particles was decreased in man after pretreatment with warfarin for more than 4 weeks The mechanism may be a direct depressive influence of "long term' warfarin treatment on the function of the R E S cells—and most probably on those which are considered a possible source of clotting factors (1, 6, 15) That "short term' warfarin treatment acts as a direct stimulant on the R E S cells seems less probable

The present results show that immune serum provokes a striking increase of the phagocytic index in all groups (Fig 2) In contrast, the phagocytic index for the same period (10½–11½ min) without injection of immune serum was found to be significantly lower than for the first recorded period (4½–8½ min) (12) The effect of immune serum is therefore even more marked than the curves (Fig 2) and phagocytic indices (Table 2) indicate An increased blood clearance of endotoxin in tolerant compared to nontolerant rabbits seems to be well documented The mechanism of the increase and of the tolerance is, however, still uncertain (7 8, 14, 16, 19) The increase seems to some degree to depend on the amount of antigen and of antibodies in serum (4, 8) In the present work the effect of immune serum supports the assumption that humoral factors contribute significantly to the increased phagocytosis in tolerant animals

There is a significant difference between the clearance curves obtained in the three groups after administration of immune serum It is, however, probable that this difference is mainly due to different concentrations of endotoxin at the time of immune injection There is no significant difference between the phagocytic rates of the groups after the injection and the shape of the curves (Fig 2) supports this assumption The results however, do not completely exclude a slight tendency towards a somewhat higher phagocytic rate in 'short term' than in 'long term warfarin treated rabbits Nevertheless, it is noteworthy that administration of immune serum abolishes, or almost completely abolishes, the difference shown between the phagocytic rates of endotoxin alone The reason for this may be that the clearance mechanism for antigen/antibody complexes may differ from that for endotoxin alone e g may involve other R E S cells and another organ distribution (4 16)

When the effect of warfarin treatment on the organ distribution of endotoxin is considered it should firstly be noted that the relative weights of some organs especially the liver, (Tables 3 and 5) are significantly lower in the two warfarin treated groups than in the untreated one In a previous experiment (12) without immune serum administration no difference was found between absolute and relative weights of warfarin treated and untreated rabbits. The difference obtained in the present work may therefore in some way be due to different changes taking place in the organs of warfarin treated and untreated rabbits after immune serum administration e g a different tissue reaction and/or a different blood flow provoked by the antigen antibody complexes (4 22)

In all groups the liver is dominant with regard to organ localization (Table 4) This organ has a considerably higher endotoxin content—both absolute and relative to other organs—than was obtained without immune serum (12) *Carey et al* (4) found a higher amount of endotoxin in the liver of tolerant rabbits—irrespective of dose and antibody titre, than in nontolerant ones They assumed the reason to be an activation and/or an increased proliferation of the Kupffer cells or a changed hepatic blood flow, rather than an antibody effect An immune serum effect however seems to be the most reasonable explanation for all groups in the present work

Moreover when present results (Table 4) are compared with previous results (12) it appears that immune serum provokes in general a considerable decrease of the amount of endotoxin in the lungs and kidneys, but has no effect on the spleen The present findings for the spleen are in accordance with *Carey et al* (4) The present lung con

tent however diverges completely from their observation (4)

They demonstrated an increased lung uptake in all tolerant rabbits irrespective of the antibody titre—also tolerant without antibodies—compared with nontolerant rabbits The lung uptake however, seemed to increase with increasing antibody titre

One explanation for the divergence between the present findings and their observation may be that activation and/or proliferation of the phagocytic cells in the lungs of tolerant rabbits will more than compensate for the immune serum effect shown in the present study The immune serum effect on the amount of endotoxin in the kidneys is noteworthy especially seen in relation to the general Shwartzman phenomenon which probably partly depends upon the amount of endotoxin in this organ (17 20) Furthermore the present immune serum provoked changes in the organ distribution pattern of endotoxin seem to be more pronounced than can be explained by only a changed organ uptake of the relatively small fraction of endotoxin cleared from blood after immune serum administration It is possible that the immune serum also provokes an immediate redistribution (2 3 5)

The organ uptake and distribution are also influenced by pretreatment with warfarin As expected from the clearance results (Fig 1 Table 2) long term warfarin treated rabbits have a significantly lower total endotoxin uptake in the organs than short term warfarin treated and untreated rabbits (Tables 4 and 5)

Moreover organ uptake values are somewhat but not significantly higher in short term warfarin treated than in untreated rabbits This is in accordance with the findings obtained in short term warfarin treated and untreated rabbits without immune serum administration (12)

The present absence of significant differences of organ content in the two latter groups which have significantly different clearance rates may be due to differences in sensitivity of the methods although a somewhat higher uptake in other organs e g the bone marrow, in short term warfarin treated rabbits is not excluded (13)

The amount of endotoxin in the lungs is significantly higher in the 'short term warfarin treated rabbits than in the long term warfarin treated or untreated rabbits No such difference was previously obtained between short term warfarin treated and untreated rabbits without injection of immune serum (12) A reasonable explanation for the different amount of endotoxin in the lungs is difficult to give Otherwise the long term' warfarin treated rabbits seem to have a slightly lower amount of endotoxin in the liver than the two other groups which may account for the lower total uptake in this group and also supports the previous assumption of a depressed function of the R E S cells in this organ (1)

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RAPIDLY GROWING MYCOBACTERIUM IN CHRONIC ABSCESS

A Case History

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In a chronic, spontaneous abscess with fistula on the thigh of a 53 year old woman, acid fast rods were found to be the only infectious agent No other apparent aetiological factors have been recorded The bacteria are rapidly growing and the colonies appear in two variations, with and without reddish pigment The strain is strongly niacin positive as are the catalase—and arylsulphatase—tests It does not ferment carbohydrates and produces a strong smell of vanilla In the course of almost 2 years, therapeutic efforts have been without success

There are few reports in the medical literature of the discovery of niacin positive, rapidly-growing mycobacteria in subcutaneous abscesses which have not occurred in connection with an injection (1, 2, 3, 5, 6, 8, 9) The object of this paper is to deal with an apparently spontaneous abscess with a chronic course, a rapidly growing mycobacterium being the only known cause This bacterium has perhaps not been described previously, but seems to have several qualities in common with *Mycobacterium borstelense* var *niacinogenes* (3)

CASE REPORT

The patient is a woman born in 1917 who has worked as a cleaner in an Oslo shoe shop during the past ten years She had pleurisy in 1951 Tuberculous aetiology was not demonstrated, but antituberculous drugs were given and since then she has had no sign of a relapse

In December 1969 she knocked her left thigh against an iron door on two occasions She remembered this trauma three months later when a painful swelling and tenderness with ensuing redness occurred in the upper lateral part of the left thigh An abscess formed and was incised several times since then it has emptied itself spontaneously 30–40 times and has not been cured until now (November, 1971)

The first biopsies did not point to any specific aetiological causes The patient was admitted to hospital in the autumn of 1970 and the abscess, with a 10 cm long fistula, was extirpated to healthy tissue three times There was a primary healing of the sore each time but spontaneous granulation from the site was not observed and the abscess returned The scar on the skin seemed to have good nourishment, but some keloid was formed Once again there was no sign of specific panniculitis resulting from the histological examination and a pus specimen gave no growth of acid fast rods The patient reported severe pains in the region of the abscess, but the cause of these pains is not clear She had no temperature during her

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stay in the hospital The sedimentation rate has for the most part been normal and not more than 25 mm/hour The total number of leucocytes has been between 4000 and 6000 In March 1971, acid fast rods were shown in a Lowenstein Jensen culture of pus from the fistula and by direct microscopy In each oil immersion field in Ziehl Neelsen smears 0–10 acid fast rods were seen—some singly and some in small heaps

No other bacteria were found in the pus by microscopic examination of Gram stained smears, or by cultivation on the customary media and aerobic and anaerobic cultivation at 37° C

The therapy, which included incision and three excisions followed by the triple treatment—SM PAS INH to begin with later ethionamide did not seem to have any effect judging by the clinical and bacteriological criteria

During a general examination in September 1971 nothing pathological was found in the inner organs X rays of lungs and the urinary tract showed nothing abnormal The patient was adipose She was approximately 5 ft 7 ins (170 cm) and weighed a little over 14 stone (90 kg) There were no signs of exanthema or other pathological skin changes apart from the scar (fistula) itself on the left thigh The scar was star shaped and the opening of the fistula had a diameter of 1,5 cm The 10 cm long fistula finished superficially with no attachment to either muscle or bone-marrow This was verified by probing and by X ray examination with contrast The fistula produced a thin yellow pus with no unpleasant smell The Pirquet reaction was slightly positive 2 3 mm after 72 hours

Cell Morphology

The acid fast bacilli are morphologically like *M tuberculosis* They are straight or slightly curved rods with rounded sometimes slightly swollen ends The bacilli are generally acid fast but in a few smears from Lowenstein Jensen cultures they are partly decolourized

Cultures

In March/April 1971, 14 pus specimens were cultivated on 5 Lowenstein Jensen slopes each and examined after 2–3 weeks in the daily routine of the laboratory Two types of colonies were observed on the primary culture media 604 colonies of type A grew on 60 slopes whilst 22 colonies of type B grew on 4 slopes from four different specimens Four slopes had no growth and two were contaminated Both type A and type B colonies grew from the same specimen but never both on the same slope Type A colonies grew most frequently in secondary cultures even from type B colonies

Type A The colonies are 2–4 mm in diameter after 2–3 weeks Young colonies are smooth spherical and with an even edge They are greyish white to beige with a paste like consistency Older colonies are concave in the middle and have a wrinkled ring shaped surface (cockade) with a diameter from 6–8 mm

Type B After 2–3 weeks the colonies are 1 2 mm in diameter They are spherical smooth greyish white to beige with a red ring around a central bud When type A colonies were diluted on a series of slopes 20 type B colonies grew on the fourth tube all of which were of the same size but only half of them had a reddish brown protuberance in the middle Cultures that had been at room temperature for 2–3 months showed growth of a reddish brown protuberance on one fifth of the tubes A few were brick red

The medium was yellowish brown under both type A and type B colonies There was a strong smell of vanilla, especially from the older colonies where a cork stopper in the tube allowed a good supply of air The strain grows at 20 30 37 and 40° C but not at 45 or 52° C The rate of growth allows the result of the sensitivity test to be seen on the Lowenstein Jensen medium after 3 days and after 2 days when the paper disc method is used on blood agar

In Bacto brain liver heart (semisolid) medium there was aerobic growth only on the surface and the strain grew like a film up inside the glass In Dubos broth base (Bacto 385) the growth sinks to the bottom leaving the rest of the broth quite clear

Sensitivity Tests

Sensitivity tests performed on Löwenstein Jensen medium showed that the strain was resistant to para aminosalicylic acid isoniazid

cycloserine capreomycin ethambutol (10), thiosemicarbazon rifampicin pyrazinamide and sensitive to ethionamide and viomycin moderately sensitive to streptomycin On media containing PAS in concentrations of 100 500 and 1000 μg/ml the colonies became brown and black—as Bonicke has shown for *Mycobacterium borstelense* (3) With the paper disc method (7) on blood agar the strain was resistant to penicillin sulphonamide chloramphenicol tetracycline erythromycin nitrofurantoin colistin carbenicillin cephalosporin ampicillin methicillin lincomycin nalidixin fucidin and gentamycin but sensitive to kanamycin

Biochemical Tests

The niacin test was strongly positive as was the catalase reaction which became negative after heating to 68° C Nitrate reduction test was positive the indol reaction negative Arylsulphatase test (14) was spontaneously strongly positive after 24 hours 48 hours and 3 weeks

Bonickes (4) series acetamide nicotinamide and pyrazinamide were strongly deaminated (7 μg/ml) carbamide benzamide isonicotinamide allantoin malonamide were slightly deaminated (2 3 μg/ml) and salicylamide succinamide were not deaminated The series were examined after 24 hours only

None of the following carbohydrates were fermented glucose lactose mannitol sucrose maltose galactose mannose sorbitol dulcitol inositol trehalose xylose raffinose starch dextrin Na malonate inulin arabinose Na citrate aesculin sorbose melibiose levulose arbutin cellobiose L-tartrate l tartrate Gelatin is not liquefied

Pathogenicity

Three guinea pigs were injected with the referred strain in a suspension of 5 μg/ml One guinea pig received 1 ml subcutaneously in the right groin another 1 ml intramuscularly in the right thigh and the third received 0 2 ml intracutaneously in a shaved skin area on the abdomen A pea sized nodule developed subcutaneously in the right groin during the first two weeks but was not palpable from the fourth week The intramuscular injection gave no palpable affection A 1 2 mm large nodule which developed intracutaneously during the first two weeks also disappeared When the guinea pigs were autopsied after 8 weeks there was no local or general sign of infection The Mantoux reaction was negative in all the guinea pigs with 0 1 ml 6 per cent OT Six white mice received injections of doses from the same suspension lot as the guinea pigs Two mice received 1 ml intravenously each 2 received 1 ml intraperitoneally each and 2 mice had 0 2 ml each injected into the paws of the hind feet They were killed after 8 weeks but no sign of infection could be observed

DISCUSSION

The woman s lesion in the left thigh seems to have followed the same pattern for over one year and a half It is therefore reasonable to suppose that the bacteria which were found represented the aetiological factor right from the beginning This is quite probable as there has been no information of other infections or any injections in the skin area concerned nor has any sign of subcutaneous atrophy in the adipose tissue been revealed in this region or in other parts of the body skin (5 6) It lasted however more than one year before acid fast rods were found in the fistula secretion. This can be explained by the fact that the atypical colonies were overlooked as they were thought to be simple contaminants Besides in the beginning very few specimens were sent in for examination of acid fast rods as the slight Pirquet reaction and the normal sedimentation rate did not point to a tubercular infection

It is tempting to compare the described strain with *Mycobacterium friedmannii* (11 12 13) which according to *Stanford & Beck* (1969) and others (6 8) is of the same species as *M abscessus M runyonii M bor*

stelense It is especially tempting to compare it with *M borstelense* var *niacinogenes* (3), since this species includes rapid-growing bacteria with smooth, butyreous, greyish white colonies with positive niacin-, catalase-, and arylsulfatase tests Gelatine did not liquefy, high PAS concentrations gave brown and black colonies on Lowenstein-Jensen media and showed resistance to ethambutol (10), but sensitivity to ethionamide The difference between *M borstelense* var *niacinogenes* and the described strain was as follows The described strain was cultivated from a chronic abscess while the former was isolated from expectorate and gastric lavage, mostly from patients with tuberculous affection The latter had some red-pigmented colonies that decolorated the medium and smelt strongly of vanilla The nitrate reduction test was positive and no carbohydrates were fermented, while the former fermented glucose, mannose, trehalose and levulose (late) The latter grew at 40° C, acetamide was de-aminated and the strain was sensitive not only to ethionamide, but also to canamycin and viomycin

The conclusion of this comparison is that the identity of the referred strain is not clear, especially because the pigmentation pattern varies from greyish-white to brick red in the centre of some colonies

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FOUR NEW *ESCHERICHIA COLI* O ANTIGENS, O148, O151, O152, O153, AND ONE NEW H ANTIGEN, H50, FOUND IN STRAINS ISOLATED FROM ENTERIC DISEASES IN MAN

With a Discussion on the Future Numbering of K Antigens

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Among *Escherichia coli* strains received recently at the International Escherichia Centre were several which could not be determined serologically by the available test sera Some of these strains had a possible causal relationship to the enteric disease from which they were isolated, and it was considered important to establish the hitherto unknown antigens as test antigens This paper reports the examination of some of such strains isolated from human disease The paper concludes with a short discussion of the present concepts of K antigens in *E coli*, explaining why new K antigen numbers were not established on the basis of the strains examined

MATERIAL

Two strains were received from *R Sakazaki*, Japan Salmonella and Shigella Centre, National Institute of Health Tokyo No 880 67 was from an outbreak of gastro-enteritis and other strains with a similar serological type have been isolated from several such outbreaks in Japan No 1184-68 was isolated from several cases of dysentery like disease and gave a positive keratoconjunctivitis test according to *Sakazaki* Thus both strains were isolated from enteric diseases in adults and children, but they were not primarily isolated from cases of infantile diarrhoea

The strain E519–66, received from *Joan Taylor*, Salmonella Reference Laboratory, Central Public Health Laboratory, London, was isolated together with many other strains of the same serotype in Aden from soldiers with diarrhoea disease acquired [illegible] (12) [illegible] the

[illegible] om *G Ruckdeschel*, Max von Pettenkofer Institut, Munich This strain and several others with the same serotype were isolated from mild cases of infantile diarrhoea in children's institutions

Medium

The D_3 medium (B) with 0 05 per cent glucose was used as solid medium (14)

METHODS

Antiserum production, agglutination techniques and procedures for absorption of antisera were per

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TABLE 1 *Biochemical Properties of Strains Examined*

	880-67	1184-68	E519-66	14097
Adonitol	—	—	—	—
Dulcitol	+	+	—	—
Sorbitol	$+^{*}$	+	+	+
Arabinose	+	+	+	+
Xylose	+	+	+	+
Rhamnose	+	$+^{8}$	+	+
Maltose	+	$+^{10}$	+	+
Salicin	$+^{4}$	$+^{13}$	$+^{2}$	—
Inositol	—	—	—	—
Lactose	—	+	+	+
Sucrose	—	—	+	+
Sorbose	$+^{13}$	—	—	—
Mannitol	+	+ +	+ +	+ +
Glucose	+	+ +	+ +	+ +
Indol	+	+	+	+
H_2S	—	—	—	—
Gelatin	—	—	—	—
Ammonium glucose	+	+	+	+
Ammonium citrate	—	—	—	—
KNO_3	+	+	+	+
Voges Proskauer	—	—	—	—
Methyl red	+	+	+	+
Urease	—	—	—	—
KCN	—	—	—	—
Malonate	—	—	—	—
Motility	+	+	—	+
Haemolysis	—	—	—	—
	O151 H50	O152 H-	O148 H28	O153 H7

$+$ = positive after one day
$+^{*}$ = positive after 2 days
— = negative after 14 days
+ + = acid and gas after one day

formed as described by *Kauffmann* (5). Precipitation by double diffusion in gel (11) was carried out in a slightly modified form using filter paper discs (15). Immunoelectrophoresis was performed according to *Scheidegger* (13).

RESULTS

The results of fermentation reactions and other biochemical tests are shown in Table 1. All strains are typical *E. coli*. Strain 880-67 is lactose-negative and does not produce gas when fermenting mannitol and glucose, and might previously have been labelled *Alcalescens dispar*.

For determination of O antigens, overnight broth cultures heated to 100° C for 1 hour were tested for agglutination in the available O test sera 1 to 147 and 149 and in the new O sera 150 and 157 presented in the accompanying paper (1). At the same time, boiled broth cultures of all established O test antigen cultures were examined in O sera produced with the strains under investigation.

1184-68 had a weak bilateral relationship to O group 3. E519-66 gave stronger and weaker cross reactions with many O groups, strongest with O1, O16, O68, O100 and O138, but it was not identical with any of these groups. Except for these results, only few insignificant reactions with low titres were detected.

The four strains E519-66, 880-67, 1184-68 and 14097 could therefore be established as test strains for the new *E. coli* O antigens

TABLE 2 *Analysis of the Nature of K Antigens by Classical Bacterial Agglutination and Absorption test*

		O serum	OK serum Unabsorbed	OK serum Absorbed by homologous culture heated to 100° C
880-67	liv *	1280	320	0
	100° C	10240	2560	0
1184-68	l v	0	80	0
	100° C	1280	1280	0
E519-66	liv	20	80	0
	100° C	2560	2560	0
14097	liv	20	320	0
	100° C	10240	2560	0

* Living suspension from D_8 agar plate, formalinized
§Suspension from D_8 agar plate heated to 100° C for 1 hour

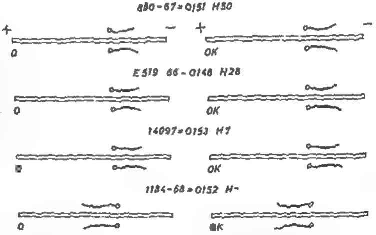

Fig 1 Immunoelectropherograms of non heated and heated bacterial extracts against homologous O and OK sera (schematic drawing) The troughs contain the homologous O and OK sera. The wells above the trough contain non heated extracts, 60° C 20 min The wells below the trough contain heated extracts *i e* the 60° C 20 min extract heated to 100° C for 1 hour

O148 O151, O152 and O153, respectively No O antigen relationships could be found between these new O antigens

In order to examine the nature of the K antigens, OK sera, *i e* antisera produced with non-heated, formalinized cultures, were absorbed with homologous cells heated to 100° C for 2½ hours (Table 2) Except for 880-67, all strains were inagglutinable in homologous O serum in the living state It will be seen from the results recorded in Table 2 that no antibodies against the live culture are left when absorptions have been carried out with boiled culture Thus the possible K antigens are not L antigens according to the classic definitions Living cultures of the five strains were examined in slide agglutination in OK sera corresponding to the established 91 K antigens, and the 91 K antigen test strains were also examined in OK

serum corresponding to the five strains under investigation Only insignificant cross reactions between the five strains and the established K test antigens were found

If the hitherto established criteria were used, the four O inagglutinable strains would be recognized as K antigen test strains for four new K antigens of the B type

In some recent publications from this laboratory the subdivision of K antigens into L, B and A antigens and the significance of these terms have been questioned According to those investigations, immunoelectrophoresis (IE) of simple extracts of E coli cultures gives a clue to important traits of the O and K antigens When the outcome of IE test is interpreted in the light of recent biochemical studies of the E coli O and K antigens it is obvious that probably only a limited number of E coli strains have special, separate polysaccharide K antigens Fig 1 gives a schematic representation of the examination of extracts of the strains in question

Both non heated and heated (100° C) extracts of strains 880 67, E519-66 and 14097 give kathodic precipitation arcs close to the application basin in O serum and in OK serum (O antigen lines) and would thus belong to IE group 1B (8) No other thermostable (or thermolabile) antigens are found indicating that these strains do not have special K antigen polysaccharides They belong therefore in IE group 1Bb in which most of the serotypes from infantile diarrhoea can be found (8) Extract of the strain 1184-68 also gives only one precipitation arc in the immunopherogram but this line which is found against both O and OK sera and both in the heated and the non heated extracts is on the anodic side of the application basin There is no separate K antigen precipitation arc This strain therefore belongs to IE group 2b and thus confirms that E coli strains from dysentery like disease giving a positive kerato conjunctivitis test belong in this IE group (8) These findings together with recent experiences with many of the E coli K antigen test strains which give similar results and in which we have not been able to demonstrate special K antigen, led to the decision not to establish these strains as new K antigen test strains

H antigens

One of the strains 880-67, did not agglutinate in any of the present 49 H test sera nor did the test antigens H1 to H49 agglutinate in an H serum produced with 880-67 880 will therefore be established as test strain for a new H antigen H50

To summarize we therefore propose the following serotype formulae for the strains examined The antigens for which the strains will be antigen test strains are underlined

880-67 O151 H50
1184-68 O152 H-
E519 66 O148 H28
14097 O153 H7

DISCUSSION

A few comments should be made regarding the new attitude proposed here towards the numbering of K antigens For many years after the K antigens were first described by Kauffmann (4) the general idea was that if an E coli strain had a K antigen it had only one just as it had only one O antigen The finding that Escherichia strains often possess fimbriae and other surface appendages (2) which, according to the classic definitions could be described as K antigens (L antigens), and that such antigens were often found together with other K antigens (10) made re evaluation of the K antigens necessary Concurrently another group of surface

capsule anti

ologically

The poly

saccharide K antigens were found in many E coli strains, and at one time we thought that capsule polysaccharides were characteristic for all E coli strains (9) Recent immunoelectrophoretic studies have shown, however that the presence of special polysaccharide K antigens is typical in the serotype strains prevalent in extraintestinal disease and in the normal intestine This

means that there are many *E coli* strains which do not possess such special thermo stable capsule antigens No special K poly saccharide has been detected in the strains frequently found in infantile diarrhoea nor can such a K antigen be found in serotype strains from dysentery like disease (8) Using the accepted terminology these strains are described as B antigen types *e g* O111 K59(B) H2 and O124 K72(B) H When such K antigens were first numbered this was done with great hesitation and they were given preliminary B numbers (6) *viz* O111 B4 H2 and O124 B17 H30 The reason for this reluctance was that there was uncertainty about the existence of a separate K antigen as long as it was not possible to produce a pure B antiserum by absorption In order to produce a pure B antiserum *e g* K59 serum another coli strain with the same O antigen O111 would be needed but with another K or with no K Such strains were never found Many new serotypes were de scribed and with a few exceptions the K antigens were of the B type As these new K antigens of the B type did not cross react with any established K test strains they were established as new B antigens B4 and B23 It was soon realized that further numbering of new B antigens would create a double set of K antigen numbers and as it was felt that no essential difference between the antigens in the old K range and the newer B range existed it was decided to give the B antigens K numbers in continuation of the already established K numbers Furthermore it was decided to stop numbering with special B numbers (7)

The recently accumulated knowledge re viewed by *Ørskov et al* (8) forms the basis for t t

u e strains hitherto established as K(B) anti gen test strains have no special K polysaccha ride specificity and the antigen which has been determined as K(B) antigen *e g* in slide agglutination should probably be con sidered to be the serological determinant of the lipopolysaccharide in the non heated bac terium *i e* the O antigen We propose there fore that numbering of new K antigens shall be limited to antigens which serologically are easily differentiated from the lipopolysaccha ride antigen We are aware that the *E coli* K antigen test strains include many that ac cording to this proposal should not have been established as antigen test strains If future investigations confirm the ideas presented such strains should probably be removed from the list of K antigen strains It should be emphasized that the experimental evidence on which the establishment of K(B) antigens was founded in practically all cases was their inagglutinability in the living state in homologous O serum This O inagglutinabili ty is often inexplicable but we think that it does not give sufficient support for the num bering of special K antigens

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TWO NEW *ESCHERICHIA COLI* O ANTIGENS, O150 AND O157, AND ONE NEW K ANTIGEN, K92, IN STRAINS ISOLATED FROM VETERINARY DISEASES

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Two *Escherichia coli* strains, one representing strains from septicaemia in chickens and the other strains from diarrhoea in piglets, were established as antigen test strains for two new *E coli* O groups, O150 and O157 One of the strains was at the same time designated as K antigen test strain for a new *E coli* K antigen, K92 *E coli* strains with O group 150 are not uncommon among human strains

Among strains of *Escherichia coli* received recently at the International Escherichia Centre (WHO), Statens Seruminstitut, Copenhagen, which could not be grouped with the available O and K test sera, two were of veterinary interest One strain was isolated from diseased chickens and the other from enteritis in piglets Both serotypes are undoubtedly of importance for diagnostic work, and the strains have therefore been established as test strains for new *E coli* antigens

MATERIAL

Strain 1935 was received at the international Escherichia Centre from *E G Harry*, Houghton Poultry Research Station Huntington, England who isolated strains of this O group from diseased chickens Strain A2 was received from *E Sweeney*, Veterinary Research Laboratory, Abbotstown, Eire, and was isolated together with other similar strains from enteritis in piglets (12) Dr *Sweeney* detected the K88ac antigen in this strain but could not determine the O antigen with the available O sera

Medium

The D5 medium with 0 05 per cent glucose was used as solid medium (10)

METHODS

Antiserum production, agglutination techniques and procedures for absorption of antisera were performed as described by *Kauffmann* (1) Precipitation by double diffusion in gel (8) was used in a slightly modified form, using filter paper discs (11) Immunoelectrophoresis (IE) was carried out according to *Scheidegger* (9)

RESULTS

The results of the biochemical tests are recorded in Table 1 Strain A2 was urease-positive, a capacity which it has in common

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TABLE 1 *Biochemical Properties of Strains Examined*

	1935	A2
Adonitol	—	—
Dulcitol	$+^{2}$	+
Sorbitol	+	+
Arabinose	+	+
Xylose	+	+
Rhamnose	+	+
Maltose	+	+
Salicin	$+^{2}$	—
Inositol	—	—
Lactose	+	+
Sucrose	$+^{2\ 8}$	+
Sorbose	+	+
Mannitol	+ +	+ +
Glucose	+ +	+ +
Indol	+	+
H_2S	—	—
Gelatin	—	—
Ammonium glucose	+	+
Ammonium citrate	—	—
KNO_3	+	+
Voges Proskauer	—	—
Methyl red	+	+
Urease	—	+
KCN	—	—
Malonate	—	—
Motility	+	+
Haemolysis	—	+
	O150 H9? H6	O157 K88ac H19

+ ~ positive after one day
$+^{2}$ ~ positive after 2 days
— ~ negative after 14 days
+ + ~ acid and gas after one day

with several other *E coli* strains isolated from the intestines of swine (5)

For the determination of the O antigen overnight broth cultures heated to 100° C for 1 hour were tested for agglutination in the established O test sera 1 to 147 and 149 They were also examined in the new O test sera mentioned in the previous paper, i e O148 O151, O152 and O153 (3) At the same time boiled broth cultures of all the O test strains were examined in O sera produced with strains 1935 and A2 Only few weak reactions were found, the strongest being low insignificant titres of A2 in O7 and O116 test sera, and of test strain O3 in O serum 1935 Therefore A2 and 1935 will be established as test strains for two new O antigens

In order to examine the nature of the K antigens, OK sera were absorbed with homologous cells heated to 100° C for 2½ hours (Table 2) Both cultures were partly agglutinable in homologous O sera when examined in the living state It was further more apparent from the results recorded in Table 2 that some agglutinins were left in the OK sera when they were absorbed by the boiled homologous culture In strain A2 these agglutinins were directed against the K88ac antigen which is an L antigen according to established conventions In strain 1935 no known L antigen was detected, but the titre remaining in the absorbed OK serum could

TABLE 2 *Agglutination of Non Heated and Heated Culture in Homologous O and OK sera*

		O serum	OK serum Unabsorbed	OK serum Absorbed by homologous culture heated to 100° C
1935	live*	80	320	80
	100° C§	10240	10240	0
A2	live	20	640	80
	100° C	10240	5120	0

* Living suspensions from D5 agar plates formalinized
§ Suspensions from D5 agar plates heated to 100° C for 1 hour

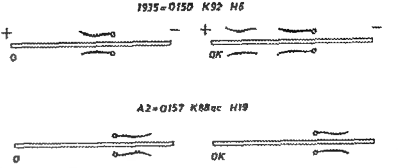

Fig 1 Immunoelectropherograms of non heated and heated bacterial extracts against homologous O and OK sera (schematic drawing) The troughs contain the homologous O and OK sera The wells above the trough contain non-heated extracts, 60°, 20 min The wells below the trough contain heated extracts, i e the 60°, 20 min extract heated to 100° C for 1 hour

be called an L titre That this L reaction is not caused by H agglutinins can be concluded from the fact that live culture from a Pril® agar plate (6) agglutinated in the L serum To make a more thorough examination of the possible K antigens non heated (60° C, 20 min) and heated (100° C, 1 hour) extracts of the two strains were examined in immuno electrophoresis against homologous O and K sera (Fig 1)

Strain 1935 (Fig 1) showed an O line directed towards the anode and, as could be expected, this was found against both O and OK sera The K line of 1935 was also anodic and was only found against the OK serum The precipitability of this antigen was not destroyed by heating to 100° C for 1 hour It is probable therefore that this K line represents an acidic polysaccharide in accordance with previous results (4) It was not clear whether the titre found in the OK serum absorbed by boiled culture was determined by remnants of the agglutinins against this polysaccharide K antigen, and only future, more intensive investigations can solve this problem

Strain A2 gave a kathodic O line and no K line in the IE test. The K88 antigen gave no line in the immunoelectropherogram According to our experience only strong concentrations of K88 antigen will give a precipitation line in IE Such a line will be located very close to the extract application well in the form of a thin but distinct concentric line

The two strains were examined by slide agglutination in the established *E coli* K antisera K1 to K91, and the test strains for these K antigens were examined in OK sera of the strains under investigation Except for the mutual agglutinations of the K88ab and K88ac antigens with OK serum corresponding to A2, no other significant cross agglutinations were detected

Strain 1935 was therefore established as antigen test strain for an *E coli* K antigen K92 As no thermostable K antigen was detected in A2, no new K number was given to that strain 1935 had H antigen 6, and A2 belonged in H group 19 The two serotypes were therefore as follows

1935 = O150 K92 H6
A2 = O157 K88ac H19

1935 will be the test strain for O150 and K92, while A2 will be the test strain for O antigen O157

DISCUSSION

In the previous paper, a brief discussion is given to justify the new attitude adopted towards recognition of new K antigens, and the reader is referred to that discussion In contrast to the strains treated in that paper, and to strain A2, we found in strain 1935 a true thermostable K antigen which is probably a polysaccharide antigen like many other K antigens primarily found among the O groups frequently present in the normal intestine and in extra intestinal disease caused by *E coli* (3) There was a low "L titre" left in the 1935 OK serum absorbed by homologous boiled culture Looking at this "L

29*

TABLE 1 *The Strains of* Moraxella *Examined and Their Origin*

Name of organism	Strain number	Origin
M nonliquefaciens	7784	NCTC[A]
M nonliquefaciens	3067/66	Oslo[B]
M nonliquefaciens	3179/66	Oslo
M bovis	3	Copenhagen[C]
M bovis	4	Copenhagen
M bovis	5	Copenhagen
M kingii	4177/66	Oslo
	= 23333	ATCC[D]
	– 10529	NCTC
M kingii	*A 1702*	*CDC*[E]
M kingii	9076/70	Oslo

A National Collection of Type Cultures, London, England
B *Kaptein W Wilhelmsen og Frues Bakteriologiske* Institutt Rikshospitalet, Oslo, Norway
C Dr K B Pedersen, The Royal Veterinary and Agricultural University Copenhagen, Denmark
D *American Type Culture Collection, Rockville* Maryland, U S A
E Center for Disease Control, Atlanta, Georgia, U S A

Electron Microscopy

The techniques were as described by *Bøvre et al* (1) *Bøvre & Frøholm* (2) and *Frøholm & Bøvre* (6) The electron microscopical examinations were usually undertaken shortly before or immediately *after the study of twitching motility*

Agar Plate Microscopy

All the strains have been examined for the existence of spreading colonies by direct agar plate microscopy of single colonies and streaks of confluent growth using the media described below If spreading ■ actually taking place the mechanism of translocation can also be determined by this method (8) A Carl Zeiss photomicroscope with phase contrast equipment and a 6 mm auxiliary condenser lens was used Observations were made with low power dry lenses (16x and 25x) Photomicrographs were taken with the photomicroscope using an electronic flash (Ukatron UN 60) and Kodak TRI X films or ADOX KB 14 films

Media

Ascites agar plates *made of two volumes of meat* extract agar (0 5 per cent meat extract 1.0 per cent peptone (Orthana), 0 3 per cent sodium chloride, 0 2 per cent primary sodium phosphate and 2 2 per cent agar) and one volume of ascitic fluid

Blood agar plates made of meat extract agar with an agar concentration of 1 8 per cent mixed with 5 per cent v/v defibrinated horse blood

All plates used were freshly poured Incubation took place at 33°C in a closed jar with ■ layer of water at the bottom in order ■ ensure a humid atmosphere Non twitching variants were observed up to 4 days before being discarded as negative

RESULTS

Colony Types and Fimbriation

SC type colonies regularly consisted of cells of which a majority had numerous fimbriae whereas the NSC type generally revealed slightly less extensively fimbriated cells and *preparations from the N type colonies showed* cells without, or with only a few, fimbriae (1 2, 6, Table 2) In the few cases where fimbriae from the N cell lines were observed *this was either coinciding* with a relatively high frequency of N to SC variation or with an unusually late development both of observable fimbriae and of corrosion beneath only large, free colonies (2, 6) Variation between the N and SC colony types, which was generally bidirectional and of low frequency, was followed by corresponding changes in fimbriation The frequency of variation appeared inconstant, and its quantitation was cumbersome due to the tendency of N colonies to outnumber SC colonies in mixed cultures on a solid medium (2, 6)

Occurrence of Spreading Colonies and Determination of Type of Translocation

The spreading zones of twitching bacteria occur around the colonies The zones are in some cases quite broad and in others almost inconspicuous Microscopically, they will be seen to consist of cells scattered over the surface in a loosely arranged lace like pattern with uncovered areas of agar in between. Individual cells or pairs of cells can be seen to move in small "jumps', covering distances from 1 to 5 μ sideways or in the direction of their longitudinal axis The number of

TABLE 2 *Correlation between Colony Type, Fimbriation, and Twitching Motility in Three Species of Moraxella*[A]

Species	Strain	Colony type[B] – cell line[C]	Degree of fimbriation[D]	Twitching motility[E]
M nonliquefaciens	NCTC 7784	N–a[F]	(—)	(—+)
		SC–a	+ +	+ +
	3067/66	N–a	—	—
		SC–a	+ +	+ +
	3179/66	N–a	—	—
		SC–a	+ +	+ +
M bovis	3	N–a	—	—
		N–b	—	—
		SC–a	+ +	+ +*
		SC–c	+ +	+ +*
		NSC–a	+	+
	4	N–a	—	—
		SC a	+ +	+ +*
	5	N–b	—	—
		SC–a	+ +	+ +*
		SC–b	+ +	+ +*
M kingii	4177/66	N–a[G]	(—)	—
		N–b	—	—
		SC–a	+ +	+ +*
	CDC A1702	N–a	—	—
		SC–a	+ +	+ +*
	9076/70	N a	—	—
		SC–a	+ +	+ +

[A] The techniques used for colony type differentiation and electron microscopical examination were as described (1, 2, 6) Twitching motility was studied by agar plate microscopy (8)
[B] SC = strongly corroding, typical with clearly visible, corroding spreading zone, in several *M bovis* variants the spreading zone was very narrow N = non-spreading and generally non-corroding NSC = moderately corroding, weakly or not spreading, often with a few more strongly corroding and spreading variants
[C] Cell line designations (a-c) correspond to those used in other publications (2, 3 6)
[D] + + = strongly fimbriated, + = slightly reduced fimbriation, (—) = fimbriae occasionally observed in the preparations, — = no fimbriae observed
[E] + + = distinct twitching motility, + = less pronounced but uniform twitching, (—+) = a few colonies exhibiting twitching observed, apparently corresponding to a variation from N to the fimbriated SC colony type (see F), — = no twitching motility detected, + +* = non twitching colonies observed, apparently corresponding to a variation from SC to N forms (2, 6)
[F] N cell line in which variation to the fimbriated state appeared with high frequency, with as many as 10^{-3} of the progeny colonies showing corroding sectors due to stable SC variants (2)
[G] N cell line showing weak central corrosion beneath large, isolated colonies only (6)

cells moving at any given moment and the number of "jumps" performed by one cell per time unit vary with the conditions and from strain to strain (8)

The results were very uniform Spreading colonies in all SC and NSC cell lines of the three species invariably showed twitching motility while signs of other spreading mechanisms were not observed, and in the N cell line colonies signs of twitching were generally absent (Table 2)

M. nonliquefaciens NCTC 7784 SC-a,

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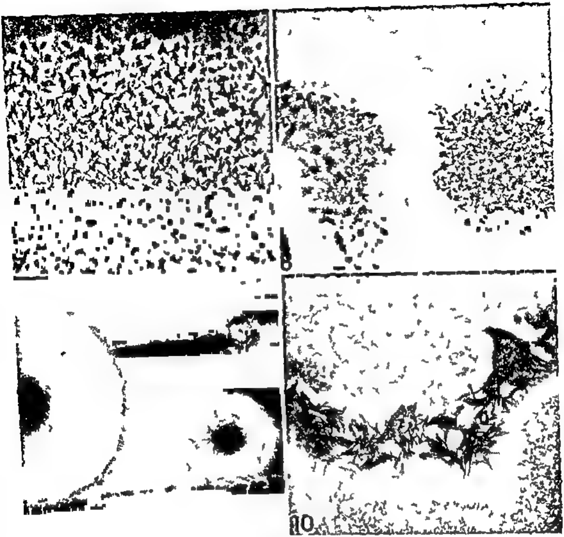

Fig. 1 *M. nonliquefaciens* 7784 SC a on ascites agar plate after 48 hours incubation in a humid atmosphere at 33 C Peripheral part of spreading zone showing a pattern typical of twitching motility A tendency to chain formation can be seen 350 ×

Fig. 2 *M. nonliquefaciens* 3067 SC a ascites agar 48 hours at 33°C 220 ×

Fig. 3 *M. bovis* 3 SC-e ascites agar 24 hours at 33 C 220 ×

Fig. 4 *M. bovis* 3 NSC a ascites agar 24 hours at 33 C 220 ×

Fig. 5 *M. bovis* 3 N-a ascites agar 24 hours at 33° C Quite pronounced chain formation 220 ×

Fig. 6 *M. bovis* 5 SC b ascites agar 24 hours at 33 C Peripheral part of spreading zone consisting also of microcolonies 220 ×

Fig. 7 *M. kingii* 4177 SC a ascites agar 48 hours at 33 C 220 ×

Fig. 8 *M. kingii* 9076 SC-a ascites agar 24 hours at 33 C 220 ×

Fig. 9 *M. kingii* 9076 N a ascites agar 24 hours at [illegible] C 220 ×

Fig. 10 *M. bovis* 3 NSC a ascites agar 24 hours at 33°C 220 ×

3067/66 SC a and 3179/66 SC a all showed pronounced i e clearly visible macroscopically, spreading of all the colonies on ascites agar plates with a microscopical picture of the spreading zones typical of twitching, including directly observable twitching motility of the cells (Figs 1 and 2) The cell lines 3067/66 N a and 3179/66 N a exhibited no twitching at all and in NCTC 7784 N a twitching colonies occurred only rarely This corresponds to the elsewhere reported instability of this N form with about 10^{-3} of the progeny colonies developing sectors or tongues of eventually stable, fimbriated SC clones (2)

M bovis 3 SC a, 3 SC c, NSC a 4 SC a, 5 SC a and 5 SC b all exhibited twitching motility on ascites agar and blood agar (Figs 3 and 4), although they gave rise to smaller colonies than the N cell lines with spreading zones that were often hardly perceptible macroscopically (see DISCUSSION) The 3 N a 3 N b 4 N a and 5 N b cell lines of this species showed neither spreading nor twitching (Fig 5) 5 SC b and some colony variants of 3 NSC a gave rise to clearly visible spreading zones comparable in size to the spreading of the *M nonliquefaciens* and *M kingii* SC forms (Fig 6)

In *M bovis* 3 SC a, 3 SC c 4 SC a 5 SC a and 5 SC b a variation to N forms with low frequency was noted corresponding to find

Although efforts were made to find just a few spreading colonies in cultures of 4 N a and 5 N b by examining thousands of colonies under the microscope such colonies were never found

M kingii 4177/66 SC a CDC A1702 SC a and 9076/70 SC a showed pronounced spreading of nearly all colonies on both agar and ascites agar plates In 4177/66 SC a and CDC A1702 SC a a few non spreading colonies occurred, however The variation of 4177/66 SC a to N like forms and of CDC A1702 SC a to stable N types has been described elsewhere (6) The spreading zones were microscopically characteristic of twitching and twitching motility of the cells was clearly seen (Figs 7 and 8) No evidence of twitching whatsoever was observed in 4177/66 N a 4177/66 N b, CDC A1702 N a and 9076/70 N a (Fig 9)

DISCUSSION

The results clearly disclose that the formation of spreading colonies by variants of *M nonliquefaciens M bovis* and *M kingii* previously shown to be closely associated with fimbriation (1, 2, 6) is also correlated to twitching motility in these organisms In all the nine strains that have been separated into fimbriated and non fimbriated cell lines (Table 2), the fimbriated cell lines form spreading and corroding colonies and exhibit twitching motility, whereas the non fimbriated cell lines form non spreading compact colonies with no signs of twitching motility microscopically Correspondingly, in cases where a variation between the SC and N colonial types is actually observable in either direction (2 6), non twitching colonies among a majority of twitching colonies or *vice versa* have been found

In contrast to the twitching *M nonliquefaciens* and *M kingii* cell lines the twitching *M* bovis cell lines 3 SC a 3 SC c 4 SC a 5 SC a and some colony variants of 3 NSC a (cf Table 2) grew smaller colonies than the corresponding N cell lines and sometimes the spreading zones could only be seen under the microscope One reason for this seemingly paradoxical result might be that apart from twitching the centrifugal pressure induced in a colony by continued cell division is another factor of great importance in the determination of colony size With these organisms that corrode the agar deeply, this pressure might very well be exerted predominantly against the wall of the pit and therefore be unable to produce the same horizontal expansion of the colony When removed from the agar each of these colonies left a hole in the agar almost the size of an entire colony In the microscope the colonies were seen to be surrounded by narrow spreading zones consisting of loosely scattered cells contrasting markedly to the

compactness of the colony proper (Fig 10)

Twitching is a special kind of bacterial motility, independent of flagella, and was first demonstrated in strains of *Acinetobacter calcoaceticus* in 1961 by *Lautrop* (13) It has also been found in strains of *Moraxella lacunata* and *Moraxella nonliquefaciens* (16), in non flagellated strains of *Pseudomonas aeruginosa* (14) and in strains of other species of *Pseudomonas* (*Henrichsen*, un published observations)

Fimbriae looking similar to those seen in *M nonliquefaciens M bovis* and *M kingii* as judged by the published micrographs (1 2 6) have also been found in *Pseudomonas* species (7) and in strains from *Thornley's* phenon 3 and 4 (20) *Ryter & Piechaud* in 1963 demonstrated filamentous appendages of similar appearance in two strains of *Acinetobacter calcoaceticus* and described them as filaments probablement d origine capsulaire (19) They thought that these filaments might be responsible for one kind of move ment that to *Piechaud* (16) appeared as if it were produced by a pull in the cell by an invisible string but they tended to relate the twitching motility as such to the cell wall structure Apparently the same kind of filaments were nevertheless later proposed to be the cause of the twitching motility of *Moraxella* and named 'proflagella (17) However no explanation of the mode of ac tion of these structures was suggested but for the allusion to flagellar activity

The presence of fimbriae must necessarily alter the properties of the cell surface and it has been demonstrated that the surface charge density and therefore also the electro phoretic mobility of type 1 fimbriated *Escherichia coli* cells is only one third to one half of that of non fimbriated bacteria (4) Twitching motility being confined to surfaces and therefore probably related to surface properties of both the substrate and the cells could very well in some way or another be influenced by the presence of fimbriae The full correlation here established between spreading by twitching and fimbriation in the examined *Moraxella* species indicates that fimbriation in these organisms is most likely a necessary condition for this kind of trans location That it cannot be a sufficient con dition in itself is known from experiments showing that a number of other factors such as the humidity of the medium and its com position are of decisive importance

Corroding and spreading colonies are also seen in *Bacteroides corrodens* (5, 9, 10, 12, 18) and judging from the published photo-graphs of the colonies and the descriptions given it seems very likely that also these bacteria form their spreading colonies by means of twitching motility In fact, *Jackson et al* have recently reported the finding of polar fimbriae possibly associated with ability to form spreading colonies in strains of anaerobic bacilli As antiserum suppresses spreading they suggest that the surfaces of the bacteria or filiform processes are im portant for the phenomenon' (11) This seems to be in close correspondence with our findings

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LOCALIZATION AND PROPERTIES OF AN ACID-SOLUBLE MUSCLE ANTIGEN REACTING WITH ANTIBODIES IN MYASTHENIA GRAVIS SERA

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Sera from patients with myasthenia gravis contain antibodies to a citric acid extractable antigen present in heart and skeletal muscle This antigen is located in the sarcolemmal/subsarcolemmal region of the muscle fibres The antigen is resistant to pH changes between 2 and 10, but is inactivated by treatment at 56° C for 60 min or at 70° C for 30 min The antigenic determinant is of protein nature

Human γG-antibodies to an acid-soluble striated muscle antigen have recently been described (*Aarli* 1972) Red cells sensitized with this antigen were agglutinated by sera from 12 out of 53 patients with myasthenia gravis The antigen was present in skeletal and heart muscle from various mammalian species and probably also in thymus No cross-reactivity occurred with smooth muscle or tissues from other organs

The present paper deals with the localization of the antigen Some physical and chemical properties of the antigen are reported

MATERIAL AND METHODS

Tissue preparations Fresh healthy skeletal muscle from blood group O individuals was obtained from the Department of Surgery

Suspensions of skeletal muscle cell membranes, devoid of intracellular proteins, were prepared as described by *McCollester* (1962)

Extraction with 0 05 M citric acid was performed as described by *Espinosa* & *Kaplan* (1968) The extract was lyophilized and the product dissolved in distilled water to a Folin value of 0 35 mg/ml (*Lowry et al* 1951) This preparation, called the acid extract, was stored in 0 01 per cent sodium azide at 4° C

Fresh rabbit heart muscle and skeletal muscle from the hind leg were taken from healthy, adult, non immunized animals The specimens were treated similarly to the human muscle

Normal human heart and uterine tissue were obtained from the Department of Pathology

Sera Sera from patients with myasthenia gravis (MG) were collected from the Department of Neurology Sera from healthy blood donors and pooled human γ globulin (AB Kabi Stockholm Sweden (Gammaglobulin 16 5 per cent Kabi®)) served as controls Precipitation and pepsin digestion of γ-globulin was performed as described earlier (*Aarli* 1970)

Rabbit antiserum to human γG globulin was prepared as described by *Larsen* & *Tonder* (1969)

Absorption of serum A suspension of skeletal muscle cell membranes was centrifuged at 1500 × g for 15 min Fifty milligrams of the wet sediment were incubated with 0 5 ml of serum MG 12, diluted 1 64 Unabsorbed serum and serum absorb-

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ed with 50 mg uterine tissue served as controls After incubation at 37° C for 1 hr, the mixtures were centrifuged at 1500 × g for 10 min The absorbed serum was tested by agglutination of red cells sensitized with the acid extract

Indirect haemagglutination and haemagglutination inhibition tests These tests were performed as described elsewhere (*Aarli* 1972)

Immunofluorescence studies Pepsin digests ($F(ab')_2$-fragments) were prepared from pooled, normal human γ-globulin, from γ globulin from serum MG 12 and from rabbit antiserum to human γG globulin The protein concentration of the digests was adjusted to the same value with all 3 samples, which were then conjugated with fluorescein isothiocyanate (kindly performed by *O Closs, M D, Bergen*)

Microtome sections of rabbit muscle, 5 μ thick, were cut in the cryostat at —20° C, fixed on glass slides in acetone at 4° C for 5 min and then dried at room temperature The sections were used within 24 hr of being prepared

In the direct test the sections were incubated for 30 min at 37° C with the conjugated ($F(ab')_2$ fragments and thereafter washed for 60 min with 6 changes of isotonic phosphate-buffered saline, pH 7 2 (PBS)

In the indirect test the sections were incubated at 37° C for 20 min with serum diluted 1 32 and thereafter washed as described above They were then incubated at 37° C for 30 min with conjugated pepsin digested antiserum, diluted 1 8, and then washed in PBS as described

The sections were incubated in 0 1 per cent Evans blue in aqueous solution for 5 min Controls, not incubated in Evans blue but otherwise similarly treated, were included The sections were washed in PBS, dried by air and covered with 10 per cent glycerol in PBS Coverslips were applied and the sections examined microscopically with a Leitz Photo-microscope with fluorescence outfit

Column chromatography One millilitre of acid extract concentrated five times was applied to a *Sephadex G-100 column (420 × 18 mm)*, equilibrated with 0 1 M phosphate buffer, pH 7 6 The preparation was eluted by the same buffer at a flow rate of approximately 20 ml/h The eluate was collected in 5 ml portions and the optical density recorded at 254 nm in a Uvicord absorptiometer (LKB produkter AB, Stockholm, Sweden)

For DEAE column chromatography, 10 ml of acid extract, dialyzed against 0 01 M phosphate-buffer, pH 8 0, were applied to a DEAE-column (350 × 18 mm), equilibrated with the same buffer Fractions were obtained with 0 01 M and 0 3 M buffers, pH 8 0 The fractions were obtained as described above

Preparative ultracentrifugation One millilitre of acid extract diluted 1 8 was applied to a 10–40 per cent sucrose gradient and centrifuged for 17 hr in a Spinco Model L 2 Ultracentrifuge at 35 000 rpm (*Kunkel*, 1960) Fourteen successive equal fractions were collected from a pinhole just above the bottom of the tubes The fractions were numbered from bottom to top and tested for inhibition of haemagglutination without further treatment since controls with 40 per cent sucrose did not interfere with the results

Paper chromatography Hydrolysis was performed with 2 ml of acid extract in 3 N HCl for 3 hr at 100° C The hydrolysates were evaporated to dryness *in vacuo* above NaOH pellets and taken up in 0 2 ml PBS Circular paper chromatograms using Whatman no 1 filter paper, were run in the following systems

A) BuOH HAc H_2O (4 1 1), B) PrOH NH_3 d 0 91 (6 4 4), C) EtAc Py H_2O (40 11 6) and D) Isopropanol 1N HCl (65 35)

Spray reagents 1) Sodium periodate – benzidine and 2) aniline hydrogenphthalate in water saturated butanol (For application and reference concerning solvent systems and spray reagents se *Grov et al* 1964) Additional developing reagent for sugar and sugar alcohols 3) Silver nitrate reagent of *Trevelyan et al* (1950)

Treatment of the antigen Enzyme digestions were performed with trypsin, lipase and pepsin (Trypsin 'Difco 1 250, Difco lab, Detroit, Mich USA Lipase Koch Light lab, Colnbrook Bucks England, Pepsin 2 × crystallized, Lot No 69B – 2000, Sigma Chemical Company, St Louis, Missouri, USA)

Trypsin and lipase digestions were performed at pH 7 4 in 1 per cent solution of the enzymes Digestion with pepsin was performed at pH 4 1 with a ratio of enzyme antigen of 1 100 (w/w) All digestions were carried out at 37° C for 4 hr Trypsin digestion was stopped by adding 1 volume of 1 25 per cent N ethylmaleimide (B D H Ltd, Poole, England) to 9 volumes of the protein solution, followed by dialysis against PBS The lipase treated sample was kept at 4° C until used Pepsin digestion was stopped by raising pH to 8 0 followed by dialysis against PBS

All preparations were tested for inhibition of haemagglutination Controls included samples incubated with PBS instead of enzyme, and samples with PBS and enzyme but without antigen.

For oxidation with periodate, equal volumes of antigen, diluted 1 4 and 0 01 M sodium – meta – periodate (E Merck AG, Darmstadt, Germany) were mixed and left at rom temperature overnight in the dark Thereafter the mixture was dialyzed against PBS and tested for serological activity

EXPERIMENTS AND RESULTS

Localization of the Antigen

In order to investigate whether removal of the intracellular, contractile proteins from the muscle fibres influenced the serological activity of the preparation serum MG 12 was incubated with sedimented skeletal muscle cell membranes After centrifugation the serum supernatant was tested by agglutination of tanned red cells sensitized with acid extract The membrane preparation removed the agglutination activity The titre remained unchanged after absorption with uterine muscle which was used as control Absorption with sarcoplasm preparation was not performed The results indicated however that removal of intracellular proteins did not eliminate the antigen from the muscle cell

The muscle cell membrane preparations were then extracted with 0 05 M citric acid Tanned red cells sensitized with this extract were agglutinated by serum MG 12 in high titres but not by normal sera These data suggest that the antigen is localized in the muscle cell membrane

In a further attempt to locate the antigen immunofluorescence techniques were applied In the direct test using conjugated F(ab) fragments from serum MG 12 the fluorescence was located to the outer parts of the muscle fibres (sarcolemmal and subsarcolemmal regions) From this border area cross striations of lower intensity traversed the lateral parts of some of the fibres These striations were more distinct in the control preparations not stained by Evans blue

In the indirect fluorescence test both rabbit heart and skeletal muscle were employed With both tissues an intense fluorescence of the sarcolemmal and subsarcolemmal regions appeared corresponding to the pattern observed in the direct test (Figs 1a and b)

Prior treatment of the sections with serum MG 12 almost abolished the fluorescence caused by the corresponding conjugated F(ab) fragments while prior incubation with poled normal human serum did not interfere significantly with the results In some experiments however, the intensity of the sarcolemmal/subsarcolemmal fluorescence seemed to increase after pretreatment with pooled normal human serum while the striational fluorescence became weaker Incubation of conjugated MG 12 F(ab) – fragments with 10 times concentrated acid extract before use in the direct immunofluorescence test caused complete inhibition of the sarcolemmal/subsarcolemmal (but not of the striational) fluorescence

With conjugated normal $F(ab)_2$ - fragments only a weak and diffuse staining appeared In the controls without Evans blue cross striations appeared but no distant sarcolemmal/subsarcolemmal fluorescence Furthermore, prior incubation of the sections with pooled normal human serum reduced this staining obtained by normal F(ab) – fragments

Data obtained by immunofluorescence tests are thus in accordance with results obtained in experiments with muscle cell membrane suspensions They strongly suggest that the antigen is located in or in close relation to the muscle cell membranes

Physico – Chemical Properties of the Extract

The acid extract was a clear and colourless fluid with pH 4 1

In order to examine the effect of pH changes on the serological activity the pH of 0 5 ml samples of the extract diluted 1 8 in saline was adjusted by addition of HCl or NaOH to values between 1 and 12 The samples were left at rom temperature for 4 hr and thereafter dialyzed against large volumes of PBS at rom temperature The different samples were then tested for inhibition of agglutination No activity was recorded after the antigen had been treated at pH 1, 11 and 12 Otherwise the antigen was unaffected by pH changes between 2 and 10

The antigen was resistant to low temperatures as the reactivity remained after freezing and storage at —20° C for several days However after storage for 4 weeks or more, the reactivity gradually disappeared

Samples of 0 5 ml of the acid extract di

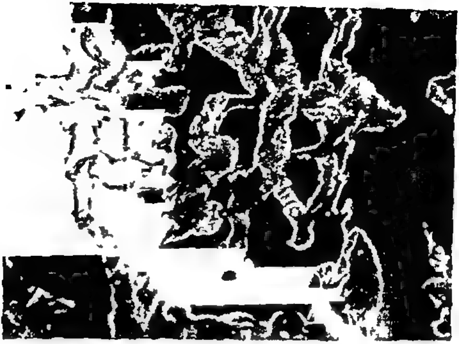

Fig 1a Indirect fluorescence Section of rabbit skeletal muscle incubated with serum *MG 12* diluted 1 32 and then with fluorescein labelled rabbit F(ab')$_2$ anti human γG globulin and with Evans blue Fluorescence of sarcolemmal and subsarcolemmal region of muscle fibres ($\times$ 210)

Fig 1b The same as Fig 1a but with greater magnification ($\times$ *1200*)

luted 1 8 in PBS, were exposed to 37°, 56° and 70° C, respectively The activity remained intact after treatment at 37° for 60 min After 30 min at 70° or 60 min at 56°, however, no activity remained Exposure to 56° for 30 min reduced the inhibition titre from 128 to 32 After ultracentrifugation of the acid extract, no serological activity was recorded with the first 9 fractions from the bottom Only fractions 10, 11 and 12 inhibited the haemagglutination Comparison with bovine serum albumin (stained by bromphenol blue) indicated a molecular weight lower than that of albumin

Gel filtration of the extract on Sephadex G 100 gave one single, broad peak, containing the antigenic activity The peak started at an elution volume of 60 ml (void volume 30 ml) The retardation of the antigen on Sephadex G 100 correlates with the ultracentrifuge data and suggests a molecular weight lower than 80 000

The acid extract was then subjected to chromatography on DEAE-cellulose The fractions were tested for inhibition of haemagglutination No serological activity was recorded with fractions eluted with the initial buffer (0 01 M) Inhibition occurred with fractions eluted with 0 3 M buffer

In chromatographic analyses of the 3N HCl hydrolysates, using solvent systems A and B and reagents 1 and 3, only one heavy band appeared, corresponding to glycerol With solvent system C and spray reagent 2, no staining occurred Negative results were obtained by spectrophotometric analysis for hexosamine by the Elson Morgan method (*Belcher et al* 1954) Accordingly, carbohydrates other than glycerol were not detected in the acid extract Purine or pyrimidine were not observed under UV light after chromatography in solvent system D

Table 1 presents the results of haemagglutination inhibition experiments with antigen samples treated in different ways Both pepsin and trypsin inactivated the antigen. The activity was also reduced after periodate treatment while treatment with lipase did not affect the serological activity

TABLE 1 *Haemagglutination Inhibition Test Effect of Enzyme and Periodate Treatment on the Activity of the Antigen*

Antigen preparation treated with	*Inhibition titre*
Pepsin	<8
Trypsin	<8
Lipase	2048
Periodate	128
PBS (control)	2048

DISCUSSION

Sera from some patients with myasthenia gravis contain antibodies to an acid extractable antigen in striated muscle (*Aarli* 1972) The present study showed that these antibodies were absorbed from the serum by skeletal muscle membranes Furthermore, the antigen could be obtained in an active form by extraction with diluted citric acid from the muscle cell membrane preparation In immunofluorescence studies, the fluorescence was localized mainly in the sarcolemmal/subsarcolemmal region of the muscle fibres This pattern became more prominent when Evans blue, which eliminates the non specific staining (*Nichols* & *McComb* 1962) was employed Moreover, inhibition of the reaction was obtained by adding acid extract to the conjugated $F(ab')_2$ - fragments These data strongly suggest that the antigen is located in close relation to cell wall structures of the muscle fibres

That antibodies in myasthenia gravis sera have a specificity for sarcolemmal/subsarcolemmal structures, is strongly supported by the results of studies by several authors Thus *Hess et al* (1962) reported that fluorescein labelled γ globulin from myasthenia gravis sera bound to the sarcolemmal region of normal skeletal muscle Furthermore, *Feltkamp et al* (1963) have pointed out that subsarcolemmal fluorescence was present with sera from nearly all their cases of myasthenia gravis, while they regarded the sarcolemmal fluorescence as of an unspecific nature *Beutner et al* (1966) examined muscle biopsies from two patients with myasthenia gravis,

both of which had high titres of circulating antibodies which *in vitro* apparently bound to striations in skeletal muscle Using immuno fluorescence technique, they found no striational fluorescence of the muscle fibres but detected binding of γ globulin in the sarcolemmal and subsarcolemmal regions Similar results have been reported by *Namba et al* (1967) Although *Strauss et al* (1960) in their first report on muscle antibodies in myasthenia gravis concentrated mainly upon the striational pattern, they described sarcolemmal fluorescence with serum from one patient with dermatomyotis

Most immunofluorescence studies with antibodies from myasthenia gravis sera have, however, been focused upon the cross-striational pattern first demonstrated by *Strauss et al* (1960) Cross-striations of lower intensity, continuous with the sarcolemmal/sub sarcolemmal structures, were also observed in the present study But the subsarcolemmal staining disappeared after inhibition with the concentrated acid extract while the striational fluorescence was not inhibited Furthermore, prior treatment of the sections with the patients serum almost completely abolished the sarcolemmal/subsarcolemmal fluorescence The striational staining was not significantly reduced Consequently, the sarcolemmal/subsarcolemmal and the cross-striational fluorescence patterns seem to depend upon different antigen/antibody systems

A cross striational fluorescence of muscle fibres can, however, also be obtained with γ-globulin from healthy, non immunized rabbits (*Strauss et al* 1966) and with Fc-fragments from normal human γ globulins (*Aarli* & *Closs* 1971) Accordingly, the cross-striational fluorescence can be produced both as a result of an antigen/antibody reaction as well as a non-specific reaction Therefore, it remains to be solved whether the same determinant in muscle fibres reacts with the Fab and Fc part of the γ globulin molecule

The association of the antigen with the cell membrane is in accordance with the results reported by *Espinosa* & *Kaplan* (1968) They demonstrated that the antigens present in the acid extract of heart/skeletal muscle are localized to sarcolemmal and subsarcolemmal structures Further analysis of such acid extracts have, however, demonstrated a multiplicity of antigenic components At least 6 antigens common to heart and skeletal muscle have been detected in acid extracts of heart (*Espinosa* & *Kaplan* 1970) Four of these were common to all species tested Although muscle antibodies in myasthenia gravis sera were absorbed by human, rabbit and guinea pig muscle tissue (*Aarli* 1972), the present data do not allow an identification of the antigen with any of the different antigenic components of the acid extract On the other hand, it may be concluded that antibodies to antigens localized in the sarcolemmal/subsarcolemmal region of heart and skeletal muscle, occur not only after artifical immunization but also in sera from some patients with myasthenia gravis

These antibodies can be demonstrated by an indirect haemagglutination technique, using tanned red cells sensitized with the acid extract (*Aarli* 1972) The necessity of treatment with tannic acid before coating indicates that the antigen is of protein nature (*Kabat* & *Mayer* 1967) This was further verified in the present work by the results of enzymatic digestion of the extract Both trypsin and pepsin treatment made the antigen inactive in haemagglutination inhibition experiments The loss of antigenic activity after periodate oxidation does not necessarily imply an additional antigenic determinant of carbohydrate nature In the first place, chromatographic analyses of the extract failed to demonstrate carbohydrates other than glycerol Besides, periodate also oxidizes hydroxyl-containing amino acids The periodate effect may therefore be explained as an effect upon protein

Antibodies to this antigen are present only in about 20–25 per cent of sera from patients with myasthenia gravis (*Aarli* 1972) Since some MG sera with high concentrations of antibodies to lyophilized muscle tissue, estimated by the antiglobulin consumption test do not react with this antigen antibodies to-

wards other muscular antigens are present in other sera Studies to examine whether this difference in serological activity is related to differences in the clinical picture, are currently in progress

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THE LOCATION OF CARBONIC ANHYDRASE IN A STRAIN OF *NEISSERIA FLAVA*

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The subcellular location of carbonic anhydrase in a strain of *Neisseria flava* was saught by testing for enzyme activity in whole bacteria, ampicillin induced spheroplasts and a fraction of cells obtained after disruption by *freeze pressing* The carbonic anhydrase activity of bacterial suspensions disappeared during spheroplast formation, but the activity of a membrane bound enzyme, succinic dehydrogenase, increased In particulate cell preparations the carbonic anhydrase activity was retained through repeating washing The results indicate that carbonic anhydrase is located in the cell wall of *Neisseria flava*

The growth of neisseriae is generally stimulated markedly by increased CO_2 tension in the atmosphere At low CO_2 concentrations several *Neisseria* species fail to grow in the presence of carbonic anhydrase (CA) inhibitors such as acetazolamide and ethoxzolamide (*Forkman & Laurell* 1965, *Sanders & Maren* 1967) At higher CO_2 concentrations no such inhibition is observed Furthermore, when *Neisseria sicca* is grown at a high CO concentration only a very low amount of CA activity is found in extracts from the bacteria (*Adler et al Lindskog et al*) The function of CA in neisseriae is not known Since CA stimulates the hydration of CO_2 the knowledge of its location within the cells is of importance for the understanding of bacterial reactions with CO_2

MATERIALS AND METHODS

Bacterial strain From a throat swab culture was randomly selected an isolate of *Neisseria* that showed a good capacity of growing in air It was diagnosed as *Neisseria flava* by biochemical reactions, colony appearence and growth capacity (*Breed et al* 1957)*)

As a reference strain was used *Neisseria sicca* 6021, the strain used by *Veitch & Blankenship* (1963) in their original work about bacterial CA.

Spheroplast formation *Neisseria flava* was grown on a shaking table over night at 37° C in 600 ml Brain Heart Infusion Broth (Difco) supplemented with 0 5 per cent Yeast extract (Difco) (*BHY* medium) Broth culture 480 ml, was centrifuged at 12 000 × g for 5 min and the supernatant medium discarded The sediment was resuspended in saline and used as inoculate for spheroplast formation Half of the sediment was inoculated into 600 ml BHY medium containing 10 per cent sucrose 0 2 per cent $MgSO_4$ and 0 1 per cent ampicillin ... nd plated ... the ... lium ... and containing the same concentrations of ... $MgSO_4$ but without ampicillin Initially and at hourly intervals, samples were taken for phase-contrast microscopy of wet specimens counting of colony forming units and for determination of enzyme activity The microscopic estimates of the

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*) Typing of the Neisseria strain was kindly performed by *Prof S D Henriksen* Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt Oslo Norway

spheroplast fraction were approximations In a control experiment with a view to excluding an inhibiting effect, if any, of ampicillin on CA activity, *N flava* was incubated at 37° C during 24 h in a phosphate buffer (0 001 M, pH 7 0) with ampicillin (0 1 per cent), sucrose (10 per cent) and $MgSO_4$ (0 2 per cent)

The counting of colony forming units (cfu) The sample was diluted 10^{-4}–10^{-8} in saline, and 0 1 ml plated on chocolate agar The plates were incubated for 24 h at 37° C in 5 per cent CO_2

Determination of carbonic anhydrase activity Forty ml of the culture were centrifuged for 10 min at 12 000 × g The pellet was resuspended in 0 5 ml saline and the CA activity of 0 1 ml portions was measured with the micromethod of *Maren* (1960) The standard error of the method is between 5 per cent and 10 per cent (*Maren* 1960) The temperature was maintained at 0° C (Heto-fng' cryostat) The flow of CO_2 through the reaction mixture was regulated to exactly 100 ml per min Quantitation was made by reference to a standard curve produced with bovine erythrocyte CA (Nutritional Biochem Co) The minimum activity measurable with this method corresponded to 1 μg CA per ml

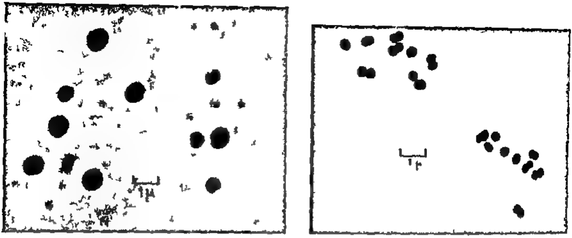
Determination of succinic dehydrogenase activity Succinic dehydrogenase is produced by neisseriae (*Jyssum* 1960) and was chosen as a marker enzyme for the cell membrane Enzymatic activity was estimated by a modification of the colorimetric method of *Nachlas et al* (1960) Two 5 ml portions of the spheroplast culture were centrifuged at 12 000 × g for 10 min The pellets were resuspended in 1 ml saline each One of the suspensions was heated in boiling water for 5 min as a blank in the enzyme assay Both suspensions were then frozen and stored at —20° C overnight before determination of enzyme activity After thawing, 0 5 ml of each suspension was added to 4 0 ml of substrate containing 0 5 ml 0 2 M sodium succinate 2 0 ml 0 1 M sodium phosphate buffer (pH 7 7), 1 0 ml 0 2 per cent 2-p-iodophenyl 3 p-nitrophenyl-5-phenyltetrazolium chloride (INT) and 0 5 ml 0 8 per cent phenazine methosulphate (PMS) (INT and PMS from Nutritional Biochem Co) The test mixture and a corresponding blank were incubated for 30 min in a 37° C water bath in the dark The reduced tetrazolium salt (formazan) was extracted by pipetting 3 ml of the reaction mixture into a glass tube containing 7 ml of acetone and toluene (5 1) After 5 sec of vigorous shaking, 0 2 ml of 99 per cent ethanol was added on the top of the mixture Within one minute the mixture separated into two layers of which the upper one was clear and contained the extracted formazan One ml of the top layer was diluted with 5 ml ethanol and its absorbancy (A_{490}) was measured in a spectrophotometer (Beckman DU) The same procedure was applied to the heat inactivated control sample

The constitution of a suitable blank for succinic dehydrogenase measurement presented the same problem as that encountered by *Nachlas et al* (1960) Replacing succinate with fumarate in the control tube was unsuitable because INT reduction occurred *N flava* supposedly contains fumarase as does *N meningitidis* (*Jyssum* 1960) Heating of the control suspension caused an unspecific increase in absorbancy exceeding that of the reaction mixture in samples taken during the first 2 h of incubation After that time the heated sample did function well as a blank

The fractionation of cells *Neisseria flava* was grown overnight in BHY medium, centrifuged, washed twice in sterile water and then suspended in 15 ml water According to absorbancy measurement (A_{540}) (Beckman DU spectrophotometer) the suspension contained, on an estimate, approximately 5×10^9 diplococci/ml A standard curve for absorbancy measurement was made by concomitant cell counting (Burker chamber) in phase contrast microscope The entire suspension was placed in a precooled (—25° C) X press (*Edebo* 1961) and the temperature of the press and the suspension was reduced to —25° C in a cooled ethanol bath The bacteria were pressed four times at pressures slightly above 2000 kp/cm² Microscopic examination showed almost complete disintegration The particulate fraction was sedimented at 48,000 × g for 15 min The CA was then measured in the supernatant solution and in the pellet The pellet was washed with three 2 ml portions of water and the CA activity measured after each washing

RESULTS

After inoculation into spheroplast broth, changes in the cellular morphology were first observed after 3 h of incubation A large number of cells increased markedly in size, yet retained their spherical shape At dilution in distilled water (1 10) they burst These large cells were considered as spheroplasts After 4 h, about 20 per cent of the cells were spheroplasts, the number increasing to about 95 per cent after 6 hours (Fig 1, 2a) Sucrose concentrations between 5–20 per cent in the broth had no influence on the time course of spheroplast formation Ampicillin concentrations as high as 0 2 per cent did not result in a higher proportion of spheroplasts $MgSO_4$ concentrations between 0 1–0 [illegible] per cent had no influence on the spheroplast formation compared with that of 0 2 per cent $MgSO_4$



a) Spheroplasts

b) bacteria of *N flava*

Fig 1 Phase contrast microscopy

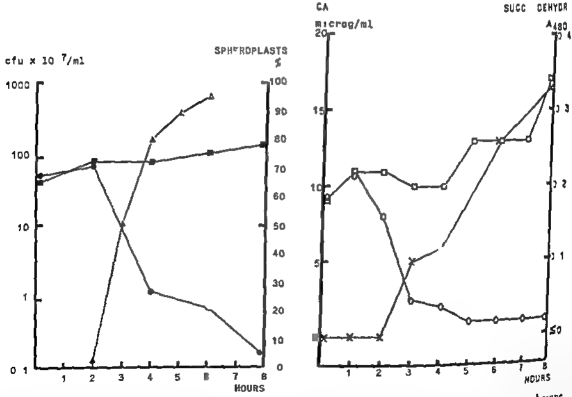


Fig 2 Spheroplast formation in spheroplast broth a) The count of colony forming units (cfu) with 0 1 per cent ampicillin (●——●) and without ampicillin (■——■) Percentage of spheroplasts with ampicillin (▲——▲)

b) CA activity corresponding microg bovine CA/ml) of concentrated cell suspension in spheroplast broth (○——○) and in spheroplast broth without ampicillin (□——□) Succinic dehydrogenase activity in spheroplast broth (×——×)

CA
microg/ml

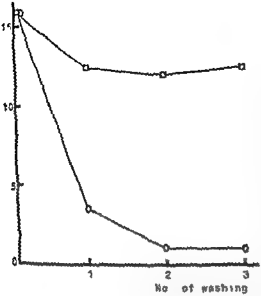


Fig 3 CA activity (corresponding microg bovine CA/ml) of *N flava* after disintegration and washing sediment (□——□) supernatant (○——○)

In all instances the cfu count decreased suddenly at the beginning of the spheroplast formation (Fig 2a)

During conversion to spheroplasts the CA activity continually decreased (Fig 2b) When about 90 per cent of the cells were spheroplasts virtually no CA activity could be found Cfu counts and CA determinations performed concomitantly revealed that the enzyme activity vanished when the cfu count was reduced to approximately 10 per cent of the initial value

In contrast parallel cultures of *N flava* in spheroplast broth without ampicillin possessed CA activity that increased during the incubation (*Fig 2b*) When the bacteria were incubated during 24 h at 37° C in a phosphate buffer with the same concentrations of sucrose $MgSO_4$ and ampicillin as in the spheroplast broth no change in the CA activity occurred The determination of succinic dehydrogenase activity at different stages of the spheroplast conversion of *N flava* showed that it increased during the spheroplast formation (Fig 2b)

After pressing a dense bacterial suspension at —25° C four times in the X press most of the cells were disrupted when preparations were examined in the phase contrast microscope Pressing reduced the absorbancy ($A_{6\,0}$) of the suspension to about one third of the original value The disintegrated suspension was centrifuged and the CA activity was determined in the supernatants and in the pellets after washing once twice and three times in 2 ml water High CA activity was found in the supernatant solution and in the pellet after the first centrifugation After the first washing CA activity of the pellet decreased slightly (Fig 3) Two additional washings did not influence the CA activity of the particulate fraction A faint enzyme activity was demonstrated only in the first washing water

The method of spheroplast formation was controlled with *Neisseria sicca* 6021 as was the CA measurement of the particulate cell fraction after disintegration This strain gave the same results as the test strain

DISCUSSION

Bacterial carbonic anhydrase was first demonstrated by *Veitch & Blankenship* (1963) in certain pharyngeal strains of *Neisseria* This enzyme is specifically inhibited by acetazolamide which also inhibits the growth of *N gonorrhoeae N meningitidis* and *N flava* but not that of pneumococci *Staphylococcus aureus Haemophilus influenzae* and *E coli* (*Forkman & Laurell* 1965) This growth inhibiting capacity of acetazolamide is dependent on the CO concentration of the growth milieu At CO concentrations of 0 5 per cent or more the growth inhibiting capacity is lost (*Forkman & Laurell* 1965 *Sanders & Maren* 1967 *Forkman* 1968) A quantitative relationship between enzyme inhibition and suppression of growth exists for many specific CA inhibitors (*Sanders & Maren* 1967) indicating that CA is essential for the growth of most

neisseriae when the environmental CO_2-concentration is low

The physiological role of CA is not known The enzyme catalyses the reaction $CO_2 + OH^- \rightleftharpoons HCO_3^-$ which is of great importance in many animal cells, e g erythrocytes and kidney tubular epithelium (*Maren* 1967) In these cases, CA facilitates the transport of carbon dioxide or bicarbonate between the surrounding medium and the cell The location of erythrocyte CA in the membrane (*Enns* 1971) seems to be ideal for this purpose Similarly, histochemical and immunofluorescence studies (*Hansson* 1965, 1968) indicate that CA is located in the cell membranes of other animal tissues Until now no report has been published on the location of bacterial CA

The presence of CA in the washed particulate cell fractions after disintegration indicates that the enzyme is located in the cell envelope Since these fractions contain cell membranes in connection with the cell walls (*Rogers* & *Perkins* 1968), working with spheroplasts was considered a method by which cell wall and cell membrane might be better distinguished

There are no earlier descriptions of methods for preparation of spheroplasts from neisseriae However, by culturing gonococci in a liquid medium *Brookes* & *Heden* (1967) found cells that were suggestive of spheroplasts L forms of *N gonorrhoeae* were produced by *Dienes* (1964) using penicillin as the transforming agent *N meningitidis* are transformed to L-forms by penicillin (*Roberts* & *Wittler* 1966 *Bohnhoff* & *Page* 1968) or ampicillin (*Roberts* 1967) In the present study, spheroplast formation was induced by incubating bacteria with ampicillin in a broth containing sucrose and $MgSO_4$

McQuillen (1956) investigated the enzyme synthesis of protoplasts by assaying the succinic dehydrogenase activity in protoplasts of *Bacillus megaterium* and observed up to five-fold increase in activity during incubation Comparable results were obtained in the present experiments with succinic dehydrogenase of *N flava* spheroplasts Originally it was supposed (*McQuillen* 1956) that the bacterial cell wall was metabolically inactive Later the concept has been proposed (*Davis et al* 1968) that the "murein sacculus" might be a giant bag shaped macromolecule which grows by a co ordinated process of bond breakage to initiate growing points, polymerization and reformation of crosslinks Such a process requires extramembraneous enzyme activities Best known of these enzymes is the transpeptidase selectively inhibited by the penicillins Similarly, the polymerizing enzymes for the polysaccharide moiety of the lipopolysaccharide component may be situated in the cell wall (*Osborn et al* 1962)

Concomitant to the conversion of the bacteria to spheroplasts the CA activity of the cells sedimented by centrifugation and resuspended to a dense suspension (80 fold concentration) decreased No CA activity was found in the non concentrated supernatant However, the sensitivity of the CA determination method is too low for unconcentrated samples The absence of CA in spheroplasts of *N flava* suggests that CA is located peripheral to the cell membrane Alternatively, the ampicillin treatment would inactivate or stimulate the inactivation of CA No direct inactivation of CA by ampicillin was observed in control experiments Stimulation of the inactivation by ampicillin of CA present in the "protoplast" does not seem likely since the site of action of ampicillin is peripheral to the cell membrane Spontaneous decrease of CA during ordinary growth did not occur in the control experiment (Fig 2) Therefore, the data available fit best with location of CA peripheral to the cell membrane

In recent years the chemical composition of the cell wall of bacteria has been used as a guide in their taxonomy (*Cummins* & *Harris* 1956) In earlier reports (*Sanders* & *Maren* 1967, *Forkman* 1968) it was noted that the only *Neisseria* strain that persistently was resistent to acetazolamide was *N catarrhalis* This observation was confirmed by *Berger* & *Ison* (1971) who found that asaccharolytic neisseriae (*N catarrhalis* *N caviae*

N. ovis, N. cuniculi) were resistent to acetazolamide and the authors proposed the exclusion of these bacteria from the genus *Neisseria*. *Henriksen & Bövre* (1968) have suggested that *N. catarrhalis* might be transferred to the genus Moraxella of the same family, *Neisseriaceae*. Since CA has been found only in neisseriae, and seems to be located in the cell wall, this might be an important taxonomic criterion for Neisseriaceae.

The need for CO_2 in the lag phase of some bacteria as suggested by *Kornberg* (1966), may be a need to form utilizable tricarboxylic acid cycle intermediates by anaplerotic fixation of CO_2. *Jyssum & Jyssum* (1970) have discussed this mode of CO_2 fixation in connection with *N. meningitidis*. Such a mechanism would certainly be favoured by the presence of CA in the cell wall.

I am greatly indepted to Prof. Lars Edebo, the Department of Medical Microbiology, Medical School Linkoping, for advice and active interest in this investigation. I wish to thank Prof. S. D. Henriksen, University of Oslo, Norway, for typing the used neisserial strain, and Docent P. O. Nyman, University of Gothenburg, Sweden, who kindly placed at our disposal the type strain *Neisseria sicca* 6021. The competent technical assistance of Mrs. Anita Lonn is gratefully acknowledged.

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PREVENTIVE EFFECT OF A NONVIRAL INTERFERON INDUCER, A BACTERIAL VACCINE, ON EXPERIMENTAL INFLUENZA IN MICE

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Intranasally inoculated Influenza A_2/Singapore virus in mice results in a pulmonary infection with dose depending mortality This experimental model was employed to study the effect of standard bacterial vaccine (SBV) on viral respiratory infection SBV, given intraperitoneally from simultaneously to 6 hours before influenza inoculation, reduced the mortality the growth of virus in the lungs, the development of gross pulmonary lesions and the seroconversion The protection appeared to be most effective, when the vaccine was given 5 hours prior to viral infection In some experiments some protection was afforded even with 24 hours or 7 days interval between administration of SBV and virus The possible mechanism of protection is discussed SBV given i p stimulates production or release of interferon (IF) Our data do not allow any conclusion whether the effect is due to production of IF or to stimulation of the immune apparatus and other nonspecific mechanisms Probably both factors are involved

The effect of bacterial vaccines in the treatment of respiratory infections has been discussed ever since its initiation many years ago Clinical comparisons between vaccine treated patients and those given placebo gave confusing results and both positive and negative reports have been published (1, 2 9, 18) It is by now well documented, that the vast majority of respiratory illnesses primarily are caused by virus and one could therefore hardly expect any preventive effect of an antibody response following administration of a bacterial vaccine During recent years, however, it has been demonstrated by numerous authors, that IF appears after bacterial infections or injections with live or dead bacteria or with bacterial extracts (3, 5, 12, 13, 15, 24, 26) The role of IF as an antiviral agent is well known The question was raised, whether the bacterial vaccines do stimulate IF production and thereby prevent viral infections In a previous paper (4) we reported that moderate quantities of circulating IF appear in the serum of mice following i p injections of a standard bacterial vaccine

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(SBV) A certain degree of protection against an experimental infection with Vesicular Stomatitis virus was also demonstrated. These findings were recently confirmed, using bacterial vaccines of similar composition. These vaccines prevented the experimental infection in mice with Semliki Forest virus (22).

In the present study an experimental model was constructed in order to examine further the protection against respiratory infections by SBV. Influenza virus was chosen for several reasons. This virus is a natural human and murine pathogen. The sensitivity of the virus to IF and IF inducers has been demonstrated by several authors, both in experimental models (19, 21) and in human epidemics (23).

MATERIALS AND METHODS

Mice. HaM/ICR/CSE/Bom albino mice were originally obtained from the National Institute of Public Health, Oslo. Young adults of either sex were used in all experiments.

Cells. The Vero line of African Green Monkey kidney cells was obtained from Dr. *J. C. Ulstrup*, Ullevål Hospital, Oslo. The cells were grown in medium 199 (Gibco) supplemented with 5 per cent inactivated calf serum and 0.044 per cent $NaHCO_3$ and maintained in the same medium but with 2 per cent inactivated calf serum and 0.132 per cent $NaHCO_3$.

Virus. Influenza A_2/Singapore 1/57 was kindly given to us by Dr. *K. Herberg*, Frankfurt a.M., Germany as an infected allantoic fluid. In our laboratory the virus was passed once in the allantoic cavity of 10 days old embryonated hens eggs. The allantoic fluids were harvested 3 days after infection. Allantoic fluids with high hemagglutination titers were pooled and stored at −70°C in 1 ml amounts. The infectious titer of the pooled virus was tested both *in vitro* and *in vivo*. *In vitro* tests were done in Vero cells employing the micromethod as described below in details. The infectious titer for mice was tested by intranasal (i.n.) administration of 0.1 ml of ten fold dilutions during light ether anesthesia. Each dilution was given to 5 mice. The infected mice were observed for 14 days and mortality was recorded daily. One 50 per cent lethal dose (LD_{50}) calculated by the method of *Reed and Muench* was $10^{5.5}$ in 0.1 ml. For each experiment a fresh ampoule was thawn and, if nothing else is mentioned, diluted in Hanks balanced salt solution (BBS) to contain approximately 10 LD_{50} per 0.1 ml.

Bacterial vaccine. The standard bacterial vaccine (SBV) was purchased from the National Institute of Public Health, Oslo. The vaccine is composed of formalin killed batceria suspended in NaCl solution and contains approx. 1.8 × 10^9 bacteria per ml. The following species are included in the vaccine: *Staphylococcus aureus, Streptococcus pyogenes, Streptococcus viridans, Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae*, and *Neisseria sp.*

Microequipment. All titrations were done in microtrays, infectivity (IT) and neutralization (NT) titrations in IS FB 96 trays (Linbro Chemical Co. Inc., New Haven, Conn.), and hemagglutination (HA) and haemagglutination inhibition (HAI) tests in Meda plates (Orion Laaketehdas Oy, Helsinki, Finland). Calibrated microdiluters and pipette droppers (Microtiter System, Cook Engineering Co., Alexandria, Va.) were rinsed in destilled water and for cell cultivation boiled for 15 min before use. The Linbro microtrays were sterilized by immersing in 96 per cent alcohol and then allowed to dry under UV light.

Tests for Biological Activities

Virus assay. Quantitative assay for the presence of influenza virus in the lungs was done by the HA test and IT test. Lungs were homogenized by a motor driven teflon grinder (Tri R model S63C, Tri R Instruments, Rockville Centre, N.Y.) in 1 ml of Hanks' BSS. The non homogenized particles were removed by centrifugation at 3000 rpm for 10 minutes and the supernatants were tested for viral activities.

For HA test two-fold dilutions of the supernatants in 0.05 ml volumes of 0.85 per cent NaCl supplemented with 1 per cent of inactivated calf serum were mixed with equal volumes of a 0.5 per cent suspension of 3 times washed guinea pig erythrocytes. The red cells were allowed to settle down at room temperature for 1-1½ hours. The highest dilution showing 50 per cent hemagglutination or more was considered to contain one hemagglutinating unit (HAu) per 0.05 ml.

For IT two fold dilutions of lung suspensions were prepared with calibrated microdiluters in 0.025 ml volumes of medium 199 supplemented with 2 per cent calf serum and 0.132 per cent $NaHCO_3$. A Vero cell suspension containing 10^5 cells per ml in the same medium kept at 37°C was added in a volume of 0.2 ml per cup with an automatic syringe. The tray was then carefully dried on the top surface with a sterile cloth and sealed with cellotape by use of a tape dispenser. The tape was firmly pressed to the surface with a tape roller. Covered in this way the tray is perfectly sealed and the tape is easily taken away if necessary for changing of medium. After vigorous shaking with a vibrator the tray was incubated for 3 days at 37°C and then examined microscopically

for development of cytopathogenic effect (CPE). Occasionally the trays had to be incubated for 4 or 5 days to get a clear titration end point. In these cases the medium was changed on the third day. The tape was removed and the medium was taken away by careful suction with a pipette connected to the water pump. With an automatic syringe 0.2 ml of fresh medium was added per cup and the tray was dried and taped as described.

Antibody assay. For titration of neutralizing antibodies in the mouse serum the same system was used as described for IT. Two fold dilutions of inactivated mouse serum were prepared in 0.025 ml volumes with microdiluters and mixed with equal volumes of influenza virus dilution containing approx. 100 tissue culture infectious doses ($TCID_{50}$). The serum virus mixtures were allowed to stand at room temperature for one hour, and then 20 000 Vero cells in 0.2 ml of medium, kept at 37°C, were added to each cup. The trays were sealed and incubated at 37°C after shaking. Microscopical readings were done after 3–4 days of incubation when the virus control titration, which was run in parallell with the test proper, showed 100 per cent CPE in the dilution one step higher than the dilution employed in the test. The highest serum dilution still neutralizing the CPE by 50 per cent or more was taken as the neutralizing titer.

For HAI test mouse sera were treated with neuraminidase (Koch Light Laboratories LTD, Colnbrook, Bucks, England) for 18 hours at 37°C in order to destroy unspecific receptors in the serum. The enzyme was then inactivated at 56°C in a waterbath for one hour. Two fold dilutions of the treated sera were prepared in 0.025 ml volumes and mixed with equal volumes of influenza virus dilution containing 4 HAu. After one hour at room temperature 0.05 ml of a 0.5 per cent suspension of guinea pig erythrocytes were added to each cup. Reading was done after 1 1½ hour at room temperature or if necessary over night at +4°C when the cells had settled down. The highest serum dilution still inhibiting the hemagglutination by at least 50 per cent was taken as the HAI titer.

RESULTS

Effect of the Vaccine on Mortality and Development of Infection

A group of 15 mice was injected intraperitoneally (i.p.) with 0.2 ml of undiluted SBV and 4 hours later inoculated i.n. under light ether anesthesia with 0.1 ml of influenza A virus containing approx. 10 LD 50. Another group of 15 mice was similarly inoculated with virus but not treated with vaccine. Four hours interval was chosen on the basis of earlier experiences (4) showing that peak titer of IF was reached 4 hours after i.p. injection of vaccine. Both groups were observed for 14 days and mortality was recorded twice a day. Mice succumbing within one day after inoculation were excluded from the experiment because death was considered to be due to manipulations during inoculation. After 14 days the survivers were bled to death through the axillary vein under ether anesthesia. Lungs were removed and inspected for macroscopical signs of pneumonia. The extent of pneumonia was quantitated using an arbitrary 0–3+ score per lobe (total maximal pneumonia score 15+ per animal). Serum from each survivor was tested for presence for HAI and NT antibodies. The experiment was repeated 3 times with the same number of mice. Fig. 1 illustrates graphically the cum-

TABLE 1. *The Effect of Bacterial Vaccine on Mortality and Development of Infection Following Influenza Inoculation (3 Experiments)*

	Vaccine group	Control group
Total number in group	43	41
Number of survivors at 14 days	25	10
Per cent mortality	47.9*	75.6
Survival in days		
Total	476	387
Mean	11.3*	9.4
Mean serum titer		
Hemagglutination inhibition $\log_2$	9.0	8.4
Neutralizing antibodies $\log_2$ §	7.0**	9.4
Pneumonia score, mean	4.65+**	9.5+

* Significant on the 5 per cent level, (mortality measured by Chi square and survival by Wilcoxon's two sample test)

** Significant on the 1 per cent level (Wilcoxon's two sample test)

§ Only examined for survivors from 2 experiments

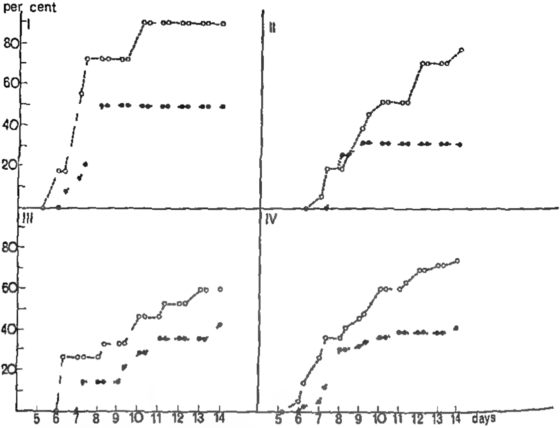

Fig 1 Mortality recorded twice a day for influenza infected mice with (●—●) or without (○—○) vaccine treatment I III describes the results from 3 parallel experiments and IV the combined results from these experiments For details see text

TABLE 2 *Effect of Bacterial Vaccine on the Quantity of Virus Obtained from the Lungs Following Influenza Virus Inoculation*

	Experiment 1**		Experiment 2 §	
	Vaccine gr	Control	Vaccine gr	Control
Number in group	10	9	10	9
Mean virus titer				
Hemagglutinins	15*	50	60*	188
Infectivity	25*	60	25*	160
Pneumonia score mean			3 7+	6 7+

* Significant on the 5 per cent level (Wilcoxon s two sample test)
** Vaccine treatment 4 hours before infection
§ Vaccine treatment 5 hours before infection

mulative mortality in these 3 experiments The vaccine treated group includes 43 mice and the control group 41 mice More mice died in the control group i all 3 experiments compared to those in the vaccine group The total mortality after 14 days of observation was significantly lower in the vaccine treated group ($0.01 < p < 0.05$)

Further results of these 3 experiments are summarized in Table 1 The mean survival

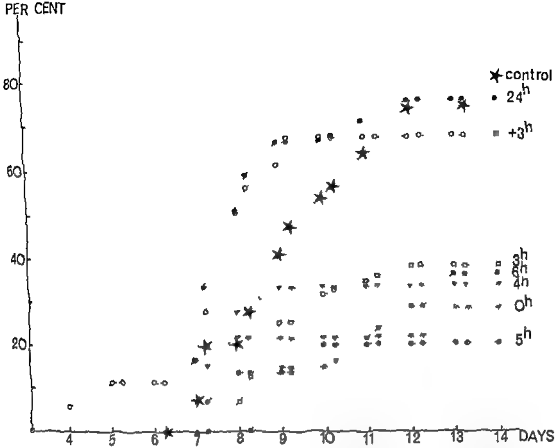

Fig 2 Vaccine effect on mortality recorded twice a day for influenza infected mice with indicated time interval between vaccine treatment and infection The $+3^h$ group was given SBV 3 hours after viral inoculation

time is significantly increased and the mean values of pneumonia score and neutralizing antibody titer are significantly lower in the vaccine treated group compared to those in the control group With an arbitrarily chosen dividing line of the quantitative extent of pneumonia we found 5 out of 25 vaccine treated mice and 7 out of 10 control mice surviving 14 days observation time having higher pneumonia score than 8+ The difference is suggestive but not quite significant ($0.1 < p < 0.05$ $\chi^2 = 3.428$)

Effect of Vaccine on Virus Replication in the Lungs

Two groups of mice, 10 in each group, were given vaccine and influenza virus 4 hours later as described in the previous experiments Five days after inoculation all mice were sacrificed, their lungs inspected for macroscopical signs of pneumonia, and pneumonia score was registered for each mouse Lungs were washed twice in Hanks' BSS, and homogenized The suspensions were tested for HA and infectivity In a repeated experiment influenza was given 5 hours after vaccine treatment The results of the two experiments are tabulated in Table 2 The mean values of HA and infectious titers in the lungs are significantly lower in the vaccine treated groups compared with the controls Two out of 20 vaccine treated mice and 15 of the 18 controls had an infectious titer of 64 or more in their lung suspensions

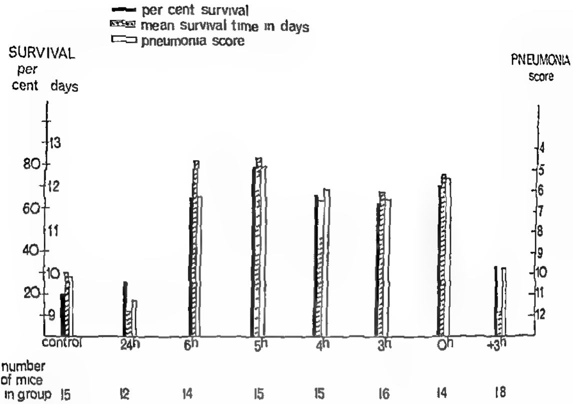

Fig 3 Vaccine effect on per cent survival mean survival time in days and mean pneumonia score in influenza infected mice with indicated time interval between vaccine treatment and infection The +3h group was given SBV 3 hours after viral inoculation

($p < 0.01$, $\chi^2 = 8.14$) The gross lung pathology was more difficult to quantitate than after 14 days as the changes were less marked The readings were especially difficult in the first experiment therefore these results were not tabulated In the second experiment there was a wide variation in the extent of gross pathological changes within both groups Negative lungs were however, only found amoung vaccine treated mice (2 mice), while heavily infiltrated lungs (pneumonia score more than 8+) were only found in the untreated control group (3 mice) The difference in the mean value of pneumonia score was not quite significant

Dependence of the Vaccine Effect on the Time Interval between Vaccine Treatment and Virus Inoculation

In order to find the optimal time interval between vaccine treatment and inoculation with influenza A virus on the mortality 8 groups of mice 12–18 mice per group were injected with 0 2 ml of undiluted SBV At various times from simultaneously to 24 hours later all mice were inoculated i n under ether anesthesia One group was given SBV 3 hours after virus inoculation The groups were inspected twice a day and deaths were recorded After 14 days the survivors were sacrificed their lungs inspected for macroscopical signs of pneumonia and pneumonia score recorded for each mice Cumulative mortality of the groups is graphically illustrated on Fig 2 The protective effect of vaccine given from 6 hours before to simultaneously is clear If SBV was given after viral inoculation or 24 hours before no protective effect was seen In a repeated experiment not included in Fig 2 significant protection was seen also with 24 hours and even 7 days time interval between vaccine treatment and viral inoculation gave almost significant ($0.1 < p < 0.05$)

protection Five hours difference seems to result in the most efficient protection Fig 3 summarizes the results from this experiment The three parameters recorded total mortality, mean survival time and development of gross pathological changes in the lungs are altered in parallel depending similarly on the time interval None of these parameters were altered by SBV treatment 24 hours before or 3 hours after virus inoculation Among the others the 5 hours interval seems to be slightly more protective than 0, 3, 4 or 6 hours, the differences are, however, small

DISCUSSION

The data indicate that treatment with the mixed bacterial vaccine, which containe the most common bacterial species occurring in the respiratory tract, affords a significant protection against experimental infection with influenza A virus in mice In a previous paper (4) we reported that the vaccine stimulates production or release of an antiviral agent with the characteristics of an interferon It seems to be reasonable to connect these two effects However, it would be much too premature to conclude, that all the protection found is due to the interferon effect. In fact the litterature on the *in vivo* effect of interferon and interferon inducers on influenza virus infection is to a large extent self contradictory Some authors reported that such a treatment to a variable extent reduced growth of influenza virus in the lungs reduced mortality and seroconversion (14, 16, 17, 20, 21, 25) Others have not been able to influence the development of experimental influenza infection (8), while in *in vitro* organ culture preparations even small amounts of IF reduced the growth of influenza virus (6) Local administration of exogenous interferon in the respiratory tract suppressed influenza growth in the lungs and development of clinical disease both *in animal* experiments (6 7) and in man (23) These experiments suggest, that IF may not pass readily from the blood into the respiratory tract secretion such as mucus Thus it is still a matter of doubt whether the *in vivo* effect is due to interferon or some other mechanism initiated by the inducer

Our experiments do not give further clarification on this question The finding of a protection, even when SBV was injected 7 days before influenza infection, indicates that a different defense mechanism from that of the IF is stimulated No IF could be demonstrated in the serum of mice 48 hours or more after SBV administration (4) On the other side, the time interval which gave maximal protection was closely correlated with that of the highest IF titers in the serum (4) Further studies are in progress in order to separate the IF effect from that of stimulation of immune apparatus and other nonspecific mechanisms

The quantities of vaccine used in our experiments are far in excess of the doses commonly employed in human therapy Comparable doses to those used for humans did not stimulate production of detectable amounts of interferon in mice (4) Therefore, at the present stage of investigation, it is not possible to draw any conclusions concerning the possibility of stimulating production of IF and its preventive effect in human beings

The usual way of vaccine administration is repeated injections of small and increasing doses at regular 1 to 2 weeks intervals Preliminary investigations, intending to mimic these conditions, indicate (*Dahl & Degre,* unpublished observations) that SBV injections in animals previously immunized with the same vaccine affords a better prevention against influenza than in animals without previous experience with the vaccine This is comparable with the reports from different laboratories indicating that immune recognition mechanisms are involved in enhanced interferon production (10, 11) Both viral and nonviral synthetic agents stimulated more interferon in previously immunized animals, compared with nonimmunized controls Repeated injections may also have stimulated nonspecific defense mechanisms Investigations on the effect of repeated injections of SBV are under progress Results from these

studies may help to clarify the mechanism of action of bacterial vaccines against viral infections

The excellent technical assistance of Miss *Signe K Ystenæs* and Mrs *Monica Skjæret* is gratefully acknowledged

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BRIEF REPORT

CELL PROLIFERATION AND CELL DEATH IN THE PERIPHERAL LYMPHOID ORGANS OF THE MOUSE

Mogens Helweg Claesson

A considerable lymphoid cell decay takes place in the thymolymphatic system as judged by supravital dye exclusion performed on lymphoid cell suspensions (1) Tritiated thymidine (3H–TdR) autoradiography has shown that the vast majority of decaying cells in the mesenteric lymph node of the mouse are derived from non DNA synthesizing lymphoid cells—probably small lymphocytes (3) *Everett et al* (1964) used repeated injections of 3H TdR and showed that small lymphocytes in the mesenteric lymph node and the spleen of the rat consist of short lived and long lived cells with life spans of about 5 days and several months respectively

The working hypothesis of the present study has been as follows Decaying cells in lymphoid cell suspensions demonstrated by means of the dye exclusion test originate from lymphocytes with different life spans It has therefore been the purpose of the present work to determine the percentage of decaying cells originating from short lived and long lived lymphocyte populations respectively

This aim has been accomplished by combining repeated injections with 3H TdR (se above) fixation of nigrosin dye exclusion (4) and autoradiographic procedures

Material and Methods The experimental animals were young adult female mice from a non inbred NMRI strain They were injected intraperitoneally with 25 μ Ci/mouse of 3H TdR (specific activity 3 0 Ci/mol Radiochemical Center, Amersham England) every 8 hours for varying periods of time (see Figs 1a and b) The animals were killed 8 hours after the last injection and the mesenteric lymph nodes (and in some instances the spleen) were removed Single cell suspensions nigrosin dye exclusion cell smears autoradiographical procedures and analysis were performed as described previously (1, 2 4)

Results and Discussion The cellular composition of the lymph node smears as well as the percentage of nigrosin stained (non viable or decaying) cells closely corresponded to results obtained in an earlier work (3) Approximately 95 per cent of the cells were small lymphocytes with cell diametres below 7 microns (as judged by ordinary H E stained cell smears) and 16 9 per cent ± 1 1 per cent*) of the cells were nigrosin stained Fig 1a shows the labelling indices of the total number of mononuclear cells (TL) the great majority were lymphoid cells) and the labelling indices of the nigrosin stained cell fraction (SL) The connection between labelling index and time after the first 3H TdR injection was fitted to straight lines of the kind *ln index = ln a + bt* where *a* and *b* are constant values The method of the least squares was used During the first 24 hours the slope of SL was steeper than that of TL but after 24 hours the slopes of the two curves were identical From 24 hours and during the last part of the experimental period the difference between the values of SL and TL was statistically significant ($P<0.01$ Students t test) The sharp break in labelling indices at 24 hours indicates that at least two populations of cells proliferate and decay in the lymph node one with a life span averaging 24 hours and one with a very long life span obviously more than 100 days as judged by the slight increase in TL (below 1 per cent/day)

The initial labelling index of all lymphoid cells (1 4 per cent) one hour after the first injection of 3H TdR reflects the proportion of blast cells which was in a state of DNA synthesis at the time of injections As the time of DNA-synthesis averages about 75 per cent of the total cyclus time of lymphoid blast cells (6 8 10 11 14) about 2 per cent of the lymph node cells might belong to this cathegory of cells The difference between the percentage of labelled cells at 24 hours (8 9 per cent) and the

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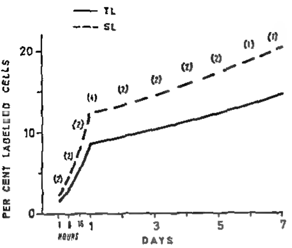

Fig 1a

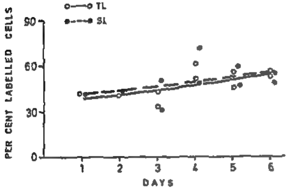

Fig 1b

Fig 1 ab Total lymphoid cell labelling index (TL) and nigrosin stained cell labelling index (SL) as a function of time after multiple injections of 3H TdR up to 7 days The numbers in parentheses reflect the number of animals at each time point In Fig b each point represents one mouse The groups of mice killed from 1 to 24 hours had received 1, 2 and 3 injections of 3H TdR respectively

percentage of lymphoid blast cells (2 per cent) gives the percentage of short lived non DNA synthesizing lymphoid cells (= 6 9 per cent) with a life span of approximately 24 hours Morphologically the majority of these cells were small lymphocytes The steeper slope of SL compared with that of TL during the first 24 hours suggests a non random cell decay among the short lived cells of the lymph node (see below), while the fact that the slopes of SL and TL are identical in the last part of the experimental period suggests a random decay of the long lived cells of the lymph node

The significant difference between SL (12 8 per cent) and TL (8 9 per cent) ($P<0\ 01$) at 24 hours might reflect a selective migration of labelled decaying cells into the lymph node The contribution of migrating non viable cells to the pool of cells decaying *in situ* might average one third as calculated from the difference between SL and TL from 24 hours and onwards Alternatively this difference might reflect a selective migration of labelled viable cells from the lymph node—or both explanations are possible

About 85 per cent of the cells in the spleen smears were small lymphocytes (see above) and the percentage of nigrosin stained cells was 31 4 per cent ± 1 9 per cent Fig 1b shows the labelling indices of SL and TL in the spleen cell smears from mice receiving various injections of 3H TdR No difference was found between the two indices The sharp increase of labelled cells during the first 24 hours followed by the slight increase from day 1 to day 6 indicates that about 40 per cent of the spleen cells have an average turn-over time of about 24 hours The slope of TL in the last part of the experimental period (2 per cent/day) suggests that the approximate turn-over time of these cells is 50 days These figures together with the identical values of TL and SI suggest that the spleen cells decay at random without respect to their age

It is believed that these results principally reflect the pattern of cell decay among lymphoid cells, since the majority of cells seen in the ILE stained smears were lymphocytes Unfortunately dye exclusion cannot differentiate between the various decaying cell types because of the rapid swelling and homogenization of the cell body during the process of stain uptake

The initial labelling of decaying lymph node cells probably reflects decay of germinal center blast cells This suggestion is in line with studies on the origin of tingible bodies (pyknotic nuclei) in germinal centers of the mouse and rat spleen and Peyer s patches (7, 8 12, 13) In these studies it was shown that many of the tingible bodies were derived from germinoblasts decaying in S—or more commonly— in G_2 phase of their cell cyclus.

A significant degree of decay might have occurred among other cell cathegories than lymphoid cells especially in the spleen namely hemopoietic stem cells and worn out polymorphonuclears.

The proportions of short lived to long lived lymphocytes seen in the present study agree well with results of earlier works performed on rats (5) but the very short life span of the short lived cell group (of which the majority are small lymphocytes) is at variance with these results This discrepancy probably reflects species differences

Conclusions The lymphoid cells in the mesenteric lymph node and spleen consist of different cell categories with respect to life span. The life span of some of these cells approximates or is less than 24 hours while other cells live for several

months Decaying spleen and lymph node cells are derived both from short lived and long lived lymphocytes In the spleen the ratio decaying cells originating from short lived lymphoid cells/decaying cells originating from long lived cells equals the ratio short lived/long lived spleen cells This is not true in the lymph node where the percentage of decaying cells originating from short lived lymphoid cells is significantly above the percentage of short-lived lymphoid cells

The findings suggest that the time of cell decay depends on the life span of the actual progenitor cell

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BRIEF REPORT

EFFECT OF A STANDARD BACTERIAL VACCINE ON INFECTION WITH AN OBLIGATE INTRACELLULAR PARASITE (*TOXOPLASMA GONDII*) IN MICE

Miklos Degré

Standardized bacterial vaccines have been used for many years for treatment and prevention of recurrent and chronical respiratory infections, in spite of the fact, that a theoretical basis of such a preventive effect has not been established Recently it became evident that a large variety of infectious agents, or extracts of such agents stimulate production or release of interferon, and prevent viral infection in susceptible cells and organisms There is also a strong indication of a preventive effect on infections with nonviral intracellular agents such as chlamydiae (4 8 13), protozoa (5, 9) bacteria (11) and fungi (6)

In a previous communication we have presented data indicating that a standard bacterial vaccine (SBV) stimulates interferon production or release in mice (2) This finding has been recently confirmed by *Singer & Hardegree* (12) Further, we have shown that SBV has a beneficial effect on the development of intranasal infection with Vesicular stomatitis virus (VSV) (2) and influenza (1) in mice The vaccine employed by *Singer & Hardegree* exerted a preventive effect on experimental infection of mice with Semliki Forest virus and parainfluenza 1 Sendai virus (Singer, personal communication) In the present experimental series investigations were conducted on the effect of SBV on experimental infection of mice with a nonviral obligate parasite, *Toxoplasma gondii*

Young adult mice of both sexes were inoculated intraperitoneally (i p) with the RH strain of *T gondii*, isolated by *Sabin* in 1941 (10) Peritoneal exudate was taken on the fourth day after inoculation Infectivity was titrated by i p injection of 0 5 ml of tenfold dilutions 0 5 ml of 10^{-3} the highest dilution resulting in 100 per cent mortality, was injected into all mice in the experimental series

Groups of mice were given 0 1 ml undiluted SBV i p and at various times from simultaneously to 14 days later inoculated with toxoplasma Each group consisted of 15 to 17 mice The mice were inspected and deaths recorded twice daily Results of several experiments employing different time intervals between SBV and toxoplasma injections are documented in Table 1 All mice died within 12 days after toxoplasma inoculation both experimental and control groups When SBV was administered from 14 days to 5 hours prior to challenge, survival of mice was slightly less than in controls When SBV was given 2 hours earlier than or simultaneously with toxoplasma, death was slightly postponed The increased survival time was highly reproducible (Table 1)

In the next experiments the effect of the dose of SBV was examined Groups of mice were given SBV i p in various amounts and 2 *hours* later challenged with *T gondii* Each group consisted [illegible] dependent to [illegible] ly [illegible] re No effect could be observed with 1/100 of [illegible] diluted SBV

The effect of the route by which SBV is administered was next determined *Groups* of mice, 15 to 16 mice in each were inoculated subcutaneously, intranasally, intraperitoneally or intravenously with 0 1 ml undiluted *SBV Two hours* later all mice were challenged with *T gondii*, and the length of survival determined as in previous experiments The mortality delaying *effect* was observed with all routes of administration Intravenous administration (*0 98 days postponement*) seems to be somewhat more efficient than other *routes* (0 38 to 0 48 days postponement)

The effect of *SBV* was compared with that of known stimulators of interferon Polyinosinic polycytidylic *acid (poly I C* Miles laboratories), 40 μg, and endotoxin (E coli O26 B6, Difco labora

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TABLE 1 *Influence of Intraperitoneally Injected 0 1 ml Standard Bacterial Vaccine (SBV) on the Length of Survival of* Toxoplasma gondii *Infected Mice Effect of Time Interval between SBV and Toxoplasma Administration*

Time interval*	Control (no SBV) Number of mice§	Control (no SBV) Mean survival time (days)	SBV-treated Number of mice§	SBV-treated Mean survival time (days)	Difference (days)
14 days (Exp 1)	15	5 60	15	5 50	−0 10
1 day			15	5 50	−0 10
5 hours			15	5 36	−0 24
2 hours			15	6 80	+1 20
None			15	6 73	+1 13
2 hours (Exp 2)	10	6 20	10	6 45	+0 25
2 hours (Exp 3)	19	6 18	15	6 46	+0 28
2 hours (Exp 4)	15	5 33	15	5 70	+0 37
2 hours (Exp 5)	15	5 80	15	6 20	+0 40
(Mean 2 hours)		(5 82)		(6 32)	(+0 50)

* Between SBV and *T gondii* injections § All mice died within 12 days

tories), 0 1 mg, were injected i p to 15 mice each, two hours prior to toxoplasma inoculation Both agents had a postponing effect on death of the same magnitude as 0 1 ml undiluted SBV (0 37 0 41 and 0 37 days respectively)

Treatment of L F1 mouse fibroblast or secondary mouse embryo cells with 0 1 ml SBV prior to inoculation with *T gondii* did not modify the development of characteristic cytopathogenic effect, nor did it influence the titre of toxoplasm

These data indicate that the bacterial vaccine influences the development of toxoplasma infection in mice after i p inoculation with *T gondii* by a slight increase of the survival time The RH strain of toxoplasma is highly pathogenic for mice, few viable organisms are *needed to develop a fatal* infection It is possible that a more pronounced effect could be obtained on a less pathogenic strain

The mechanism by which the SBV acts is not clear The data strongly indicate that the effect is not due to a localized process, as SBV introduced by different routes had a similar effect It has been demonstrated earlier that exogenous interferon modifies toxoplasma infection (9) A general nature of interferon action on intracellular protozoal infection both *in vitro* and *in vivo* has been suggested by demonstration of a similar effect on other protozoal infections (5) Stimulation of interferon production by SBV should be considered as a possible mechanism of action Interferon is produced maximally 2–8 hours after SBV injection (2) This time interval is comparable with the magnitude of effect on toxoplasm infection, and also with the strong dependance on the timing of inoculations Poly I C, endotoxin and SBV postponed the mortality to the same extent with the doses here employed Since the capacity of these agents to stimulate interferon production is widely different, it is questionable whether the titre of interferon is the limiting factor in the modifying effect on toxoplasma infection SBV did not influence the development of toxoplasma infection in *in vitro cell* cultures, nor does it stimulate production of interferon in this system (2) The possibility cannot be excluded at the present stage of investigation that alternative mechanisms, like unspecific stimulation of immune apparatus are responsible for the effect.

Interferon has been shown to influence infections with chlamydiae, rickettsiae, bacteria, fungi, protozoa and virus (14), it has some effect on tumour growth of viral and nonviral etiology, as well as on the multiplication of both tumour and normal cells (3, 7, Lindahl, personal communication) The same spectrum of effects is also observed after application of the variety of interferon inducers It seems to be obvious that the original definition of interferons needs some amplification

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PHAGOCYTOSIS OF MUTANTS OF *SALMONELLA TYPHIMURIUM* by RABBIT POLYMORPHONUCLEAR CELLS

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The engulfment rates of a series of mutants derived from *S typhimurium* 395 MS were followed by exposing heat killed ^{51}Cr labelled bacteria to rabbit peritoneal polymorphonuclear leucocytes (PMN) The mutants studied possess different lengths of lipopolysaccharide (LPS) chains on their surface Phagocytosis was generally more rapid for mutants with phage patterns typical of shorter LPS chain lengths but as in the case of virulence the presence of O specific LPS on the cell surface overshadowed the effect of chemotype The roughest mutant which possesses the shortest chain and is most liable to phagocytosis was attached by simple contact between bacterial and phagocyte surfaces, serum was of minimal importance In contrast engulfment of bacteria with O specific LPS was considerably enhanced by heat labile serum factors, probably those of the complement system The susceptibility of rough mutants to phagocytosis is discussed in terms of the interaction of hydrophobic groups on the bacterial and phagocyte surface leading to cell to cell adhesion and eventual engulfment

The structure of the cell surface is of great importance for the virulence of many pathogenic micro-organisms This has long been recognized for Salmonella (*Roantree* 1967), although the details of the mechanism are not known

The phage pattern sugar composition and immunochemistry of a series of rough mutants derived from *Salmonella typhimurium* 395 MS are well known (*Holme et al* 1968 *Lindberg & Holme* 1968) When the virulence for mice of these mutants was tested (*Edebo & Normann* 1970), the one with the shortest lipopolysaccharide (LPS) chain a glucosyl I transferaseless mutant, was the least virulent The virulence of the other mutants varied in a manner not closely correlated with phage pattern and chemotype The most virulent mutants, however, were those with O antigenic LPS on their surface

Phagocytosis has a key role in host resistance to bacterial infection Differences in virulence among strains of bacteria should be reflected in ease or difficulty of successful phagocytosis Since the efficiency of the phagocytic process, particularly at the engulfment step, is dependent upon the surface properties of the particles to be ingested, changes in the LPS of mutants should confer sufficient surface changes of the bacteria to alter their interaction with phagocytes

The experiments described in this paper were designed to examine the uptake of these well characterized Salmonella rough mutants by focussing attention on the critical engulfment step of the phagocytic process Such experiments offer the opportunity to isolate the primary interaction between phagocytes

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and essentially inert bacterial cells that in principle differ only in their exterior surface properties

MATERIALS AND METHODS

Strains The smooth strain *Salmonella typhimurium* 395 MS, and the rough mutants derived from it (R0–R10), have been described earlier (*Holme et al* 1968, *Lindberg & Holme* 1968, *Edebo & Normann* 1970)

Cultivation All strains were kept at 4°C on agar slants before use Their phage pattern were regularly checked The bacteria were inoculated into 10 ml nutrient broth (Difco) and incubated at 37°C for 18 hours Then, 1 ml was transferred into [illegible] ml fresh medium and incubated for another 4 hours at 37°C on a rotary shaker The bacteria were harvested by centrifugation (6000 × g), heat killed at 56°C for 1 hour, washed twice in phosphate buffered saline solution, pH 7 2 (PBS), and tested for sterility The concentration of bacteria was estimated with a Turner spectrophotometer (650 nm)

Labelling A modification of a method described by *Nordenfelt* (1970) was used A pellet of heat killed bacteria (5×10^9 bact) was suspended in 1 ml of $Na_2{}^{51}CrO_4$ (100 μC/ml, AB Atom energi Studsvik Sweden) plus [illegible] 25 ml 4 5 per cent NaCl and allowed to stand at 37°C overnight The cells were then washed three times in PBS Label activity of the last supernatant fluid never exceeded 2 per cent of that of the pellet The radioactivity count per cell was in the range of 10^{-4} counts per minute (cpm) A gamma scintillation counter with a NaI crystal (Picker Nuclear, White Plains N Y USA) was used

Polymorphonuclear leucocytes (PMN) were obtained [illegible] described by *Hirsch* (1956) A hundred millilitres of a 0 1 per cent glycogen solution (Nutritional Biochemical Cooperation Cleveland, Ohio USA) was injected intraperitoneally into rabbits Four hours later 150 ml saline solution containing heparin (final conc 20 IU/ml, Vitrum, Stockholm Sweden) was injected by syringe

Large numbers of cells were harvested by abdominal tap with a disposable blood donor kit Most of the collected cells were PMN when examined by microscope, after staining with Turk s reagent The exudate was centrifuged (200 × g 5 min) and the cells washed once with heparinized saline solution, and once in the phagocytosis medium The cells were counted in a Burker counting chamber Viability was tested with trypan blue stain

Phagocytosis media The basal medium used was Krebs Ringers phosphate buffer (pH 7 2) with [illegible] mM glucose (KRG *Roberts & Quastel 1963*) To test the phagocytosis promoting effect of serum components, rabbit pre immune serum, calf pre colostral serum (from the jugular vein) and bovine serum albumin (*Povivet, Amsterdam Holland*) were used in different series of experiments The blood was clotted, centrifuged and the sera split in aliquots and immediately stored at 80°C until used

Phagocytosis tests Six cellulose filters (Millipore HAWP, diam 13 mm) were attached with vaseline to the bottom of a 5 cm plastic petri dish (Nunc Roskilde, Denmark) Then 2 5 × 10 PMN suspended in 3 ml medium, were added After incubation at 37°C for one hour, most of the cells had sedimented and adhered to the filters and bottom of each dish Those not adhering were removed by rinsing with KRG Approximately 10^6 cells adhered to each filter Five millilitres of a suspension containing $2 0 \times 10^8$ ^{51}Cr labelled bacteria per ml were added to each dish The preparations were incubated at 37°C on a rocking table After 30 60 and 120 min, two filters were removed and washed thoroughly in cold saline solution The radioactivity on each filter was measured separately The fraction of bacteria engulfed by the leucocytes on one filter was calculated from the number of counts of that filter divided by the counts of the bacterial suspension The maximum uptake after two hours by one filter (obtained with the mutant R10), was around 3 per cent meaning a total uptake of about 37 per cent This assumes that the uptake was equal over the entire petri dish bottom Since the phagocytosing capacity of individual preparations of leucocytes varied, phagocytic index was used as an expression of the relative phagocytic activity This was calculated as the fraction of bacteria engulfed divided by the corresponding fraction of R10 suspended in rabbit pre immune serum subjected to the same leucocyte preparation and measured after two hours The number of R10 cells on one filter after two hours (phagocytic index = 1) varied between 1 and 3×10^7 bacteria Thus the maximum average content was 10–30 bacteria per phagocyte

RESULTS

Exchange of label between bacteria, medium and leucocytes The loss of activity from labelled bacteria to the medium in the presence of serum was routinely tested with all bacteria at the beginning of the investigation It was less than 10 per cent within 2 hours of incubation The incorporation of free chromate into the PMN was tested by mixing PMN with non labelled bacteria (R10 RO) in the presence of serum and $Na_2{}^{51}CrO_4$ (25,000 cpm) The uptake was less than 1

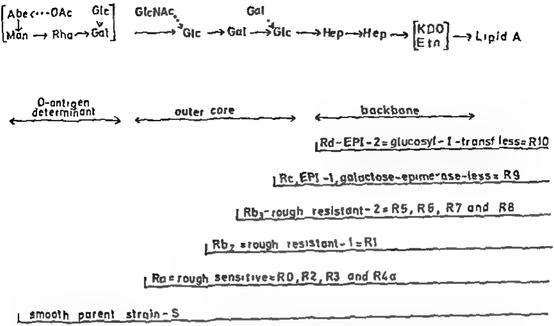

Fig 1 Proposed structures of the LPS of *S typhimurium* The lower part of the figure shows the relation between structure and chemotype (smooth Ra–Rd), phage pattern (smooth, rough sensitive etc) and the strains used in this study (S, R0 R10) A dotted line indicates that not all the molecules carry the indicated substituent Abbreviations OAc, O acetyl group, Abe, abequose, Man D mannose, Rha L rhamnose, Glc, D glucose, Gal, D galactose, GlcNAc, N acetyl D glucosamine, Hep, L glycero D manno heptose, KDO, 2 keto 3 deoxyoctonate Etn, ethanolamine (For types of bonds, see *Lindberg* 1971 see also *Osborn* 1969)

per cent The non specific adsorption of bacteria to the filters in the absence of leucocytes was so small that, for most mutants, it only slightly deviated from the background counting For R10 it was less than 10 per cent of the uptake in the presence of leucocytes Upon microscopic observation of leucocytes after rinsing, virtually all bacteria appeared to be intracellular

Phagocytosis of Salmonella typhimurium 395 MS and its mutants strains (R0 R10)

A In the presence of normal rabbit serum To stimulate the phagocytic process, normal rabbit serum was added to the medium (final concentration, 5 per cent) When the different bacteria were tested under these conditions, the engulfment differed (Fig 2) Mutants R7, R8, and R10 (the least virulent one), were most easily ingested, closely followed by R0, R1, R2, R3, R5 and R6 The parent smooth strain (MS) was the most resistant, R9 and R4a were also poorly engulfed

B In the presence of precolostral calf serum Specific antibodies might be present in normal rabbit serum and thus effect the phagocytosis of the different mutants to varying degrees To reduce this possibility, the rabbit serum was replaced by precolostral calf serum at the same final concentration (5 per cent) The engulfment of all the mutants was less in precolostral calf serum (Fig 3) than in normal rabbit serum (Fig 2) The depression was most conspicuous for the most virulent bacteria (MS R4a and R9) The order of sensitivity to phagocytosis was similar in both media

C In the presence of albumin To eliminate opsonizing serum factors altogether, the serum was replaced by bovine serum albumin at a final concentration of 0 1 per cent (Fig 4) This concentration of albumin gave the

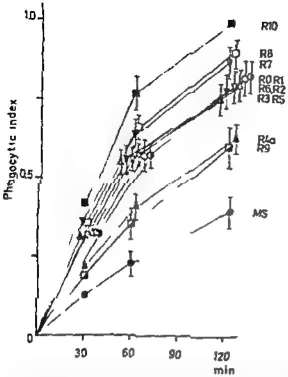

Fig 2. Phagocytosis of *S. typhimurium* 395 MS and R0–R10 in KRG containing pre-immune rabbit serum (final conc. 5 per cent). Vertical bars show standard error of the mean S/√n for five different experiments.

same uptake of R10 as did 0.2 and 0.3 per cent. The phagocytosis of all the mutants was less than that obtained either with normal rabbit serum (Fig. 2) or precolostral calf serum (Fig. 3). Particularly the phagocytosis of R5 but also that of R0, R2, R7 and R8 was reduced in comparison to that in precolostral calf serum.

In Fig. 5, data from Figs. 2–4 are rearranged to show the influence of different media on representative bacteria. MS is the smooth parent strain. R4a has moderate virulence, R10 is avirulent (*Edebo* & *Normann* 1970). With these representatives as with all bacteria tested the phagocytosis was most efficient in normal rabbit serum, then followed precolostral calf serum. Least efficient was 0.1 per cent bovine serum albumin. The impairment of phagocytic index for R10 from pre-immune rabbit serum to precolostral calf serum was approximately the same as that from precolostral serum to 0.1 per cent bovine serum albumin. The impairment of phagocytic index for R4a from pre-immune serum to precolostral serum was ap-

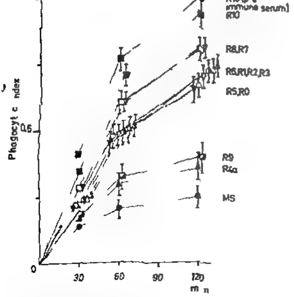

Fig 3. Phagocytosis of *S. typhimurium* 395 MS and R0–R10 in KRG containing precolostral calf serum (final conc. 5 per cent). Symbols see Fig. 2.

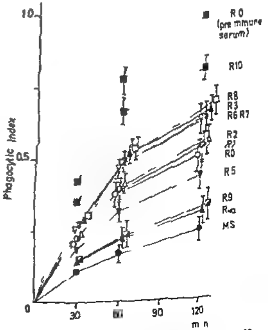

Fig 4. Phagocytosis of *S. typhimurium* 395 MS and R0–R10 in KRG containing 0.1 per cent bovine serum albumin. Symbols see Fig. 2.

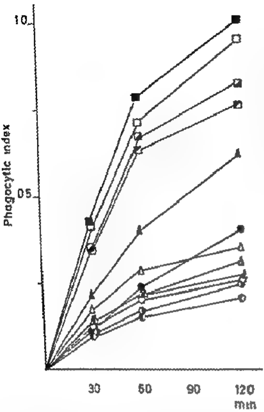

Fig 5 The dependence of the phagocytosis of *S typhimurium* 395 MS R4a and R10 on different serum components Phagocytosis medium containing pre immune rabbit serum (final conc 5 per cent) filled symbols, precolostral serum (final conc 5 per cent), open symbols, 0 1 per cent bovine serum albumin right side of symbols filled without serum components left side of symbols filled R10 squares R4a triangles S circles

proximately 50 per cent whereas the reduction from precolostral serum to [redacted]1 per cent albumin was moderate Heating of the normal rabbit serum to 56° for 30 min reduced the phagocytosis of R4a to the level of albumin MS also showed a clear drop of phagocytic index from pre immune serum to precolostral serum The difference between precolostral serum and 0 1 per cent bovine serum albumin was inconspicuous Omission of albumin only slightly reduced the index For R10 it was 0 75 in two hours for R4a 0 25 and for MS 0 2 The lowest phagocytic index encountered was 0 2 per hour

The relationship between phagocytosis and virulence for mice is shown in Fig [redacted] The sum of the phagocytic indices of each strain shown in Figs 2–4 are plotted against the surviving fractions in two groups of mice One group was challenged with 10^4 bacteria suspended in saline the other with 10^2 bacteria suspended in 5 per cent mucin (*Edebo & Normann* 1970) There is [redacted] correlation between resistance to phagocytosis and virulence, but exceptions exist R5 for example although relatively resistant to phagocytosis is almost avirulent, particularly in the absence of mucin

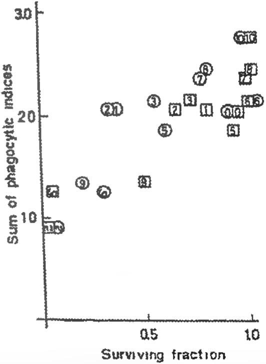

Fig 6 Relationship between virulence and phagocytosis The surviving fraction after i p injection into mice of 10^4 bacteria suspended in saline (□) or 100 bacteria suspended in 5 per cent gastric mucin (○) was plotted against the sum of the phagocytic indices in the different media containing pre immune rabbit serum precolostral calf serum or 0 1 per cent albumin Mutant number shown within symbol

DISCUSSION

In phagocytosis different consecutive steps are involved of which attachment, engulfment and intracellular processing are the main functions Each such function consists of a series of reactions In particular the intracellular processing encompasses a series of structural enzymatic and non enzymatic events which culminate in the phagocytosed particle s destruction

Several different techniques have been designed to study the uptake of micro organisms by phagocytes All of these aim to enumerate the number of microbes bound to or free from the phagocytes Microscopic counting is often used to distinguish free attached engulfed and digested particles but is laborious and quantitatively inexact Separation of phagocytes from free microbes and quantitation of either or both of them makes possible a calculation of attached plus engulfed microbes For such quantitation viable counts and radioactivity measurements of labelled microbes have been used In spite of adequate controls the viable count method carries the risk that some microbes may have multiplied or may be dead Activities of living microbes also effect the uptake Radioactivity measurements of labelled living microbes do not discriminate multiplication or death but are influenced by the effects of the living microbes on the phagocytes Since the effect of molecular differences in the cell wall LPS on the engulfment process was under scrutiny in this study labelled dead bacteria were used These were killed at 56°C for 1 hour which does not release substantial amounts of toxic and immunogenic material from the cells (*Edebo* & *Holme* 1965 *Nakano* & *Saito* 1968)

Peritoneal exudates elicited in rabbits by glycogen have been used as a rather homogenous source of PMN (*Hirsch* 1956) Though the phagocytic behaviour of macrophages is similar to that of PMN it is not identical (*Rabinovitch 1968*) Furthermore phagocytes elicited with the aid of an irritant or inflammatory agent are larger show heightened pinocytotic activity, differ in content of hydrolytic enzymes and may be more active in phagocytosis than unstimulated ones (*Rabinovitch* 1968) Consequently the present results might not be applicable to all phagocytic systems particularly with regard to the absolute values However demonstration of relative differences between the mutants is favoured by a homogenous population of phagocytic cells The PMN used were morphologically uniform and the phagocytosed particles seemed to be evenly distributed over the PMN population which agrees with earlier observations (*Brandt* 1965) The method did not distinguish between attachment and ingestion But since few or no bacteria were observed at the phagocytic surface the attachment was assumed to be the rate limiting step

In all suspending media tested *S typhimurium* 395 MR10 was most rapidly engulfed MS was least engulfed R10 has the shortest LPS side chain and is the least virulent mutant MS is the parent smooth highly virulent strain *(Edebo & Normann* 19 0) When the mutants are arranged according to liability to phagocytosis in the different media, generally the same order was obtained (presumed chemotype within brackets see Fig 1), where $\gg$ means great difference $>$ slight difference R10 (Rd) $\gg$ R7 (Rb_3) R8 (Rb_2) $>$ R3 (Ra) R6 (Rb_3) $>$ R0 (Ra) R1 (Rb) R2 (Ra) $>$ R5 (Rb_3) $\gg$ R4a (Ra leaky) R9 (Rc leaky) $\gg$ S (smooth) Consequently mutants with a short LPS chain tend to be more sensitive to phagocytosis than those with longer chains The same general relationship was observed when one mutant of each chemotype (Ra Re) from *S typhimurium* LT2 was subjected to phagocytosis by guinea pig macrophages and PMN (*Friedberg* & *Shilo* 1970) The Ra mutant (TV 119) was more resistant than the other mutants in all tests Phagocytosis was accomplished by mixing suspensions of bacteria and phagocytes in a medium containing 10 per cent serum from newborn calf Ingestion and killing were determined by viable counts The Ra mutant was only *slightly more* resist

ant to engulfment but considerably more resistant to intracellular killing in comparison to the other R mutants

Dlabač (1968) studied the clearance of mutants of *S typhimurium* LT2 in perfused rat livers The uptake of three Ra mutants M R_4 and R_7 was 31 9, 63 5 and 56 6 per cent respectively That of three Rb strains R_1 R_{10} and R_{18} was 60 3 73 2 and 75 5 per cent That of LT2 M1 (Rc) was 85 5 per cent Excluding the 'leaky' M_2 strain Ra strains were usually more resistant than Rb strains which in turn were more resistant than the Rc strain The only exception was the Ra strain, R_4 In similar experiments clearance from the blood of mice of intravenously injected mutants of *S typhimurium* was more rapid for the Ra mutant TV 119 than for other mutants with shorter LPS chains (*Nakano & Saito* 1968) Methodological differences and choice of mutants may account for these discrepancies Our hypothesis is that all sugars of the LPS contribute to resistance towards engulfment by phagocytes This predicts that the smooth bacteria are most resistant and the rough mutants then follow in approximately alphabetical order However qualitative and quantitative differences between individual mutants of the same chemotype derived from the same strain which show up as differences in the molar ratios of the core sugars within the chemotypes influence the engulfment by phagocytes For example the molar ratios of glucose and galactose in the Ra mutant R3 are only half of that of most other Ra mutants (*Lindberg & Holme* 1968) This deficiency might contribute to the relative sensitivity of this mutant The influence of other cell wall substances e g proteins has not been studied In addition leakage of the enzymatic defect leading to roughness known to exist for R9 R4a and R5 seems to contribute markedly to phagocytosis resistance (Figs 2-4) This was not observed in the mouse clearance tests with the two leaky mutants SL 1035 and SL 1036 (*Nakano & Saito* 1968) Non sedimentable 0 specific polymer in the supernatant fluid of ultracentrifuged (105 000 × g) phenol water extracted bacteria is demonstrable in R1, R3 R5, R6, R7, R8 and R10 (*Lindberg* 1971) It does not seem however, to enhance phagocytosis resistance Furthermore, one of these strains R6, contains substantial quantities of 0 specific material on its outer surface (*Edebo & Normann* 1970), without a conspicuous effect on its phagocytosis resistance

Studies of phagocytosis by macrophages suggest that at least two different types of receptors exist at the cell surface one a non-specific type with affinity for hydrophobic structures the other for antibody sites A third kind of receptor may play a role when complement participates (*Rabinovitch* 1968 *Mäler* 1970) Five per cent normal rabbit serum always produced the greatest phagocytosis (Fig 2 Fig 5) In 5 per cent precolosteal calf serum phagocytosis was reduced (Fig 3), and in 0 1 per cent bovine serum albumin it was further reduced (Fig 4) The phagocytosis of R10 was greatest in all media and only moderately impaired in the absence of serum Since the LPS of R10 has the shortest polysaccharide chain length (Fig 1) it is thought to be most hydrophobic The hydrophobic nature of the LPS affects the over all character of the bacterial surface A hydrophobic type of receptor seems to be sufficient for efficient phagocytosis of R10 In contrast the phagocytosis of MS R4a and R9 was markedly decreased when normal rabbit serum was changed to precolostral calf serum This suggests participation of natural antibodies in the phagocytosis of these bacteria Normal rabbit serum contains Forssman antibodies and a cross reaction between the Forssman antigen and Salmonella factor 0-5 has been demonstrated (*Jenkin* 1963) Since the mutants contain different amounts of 0 specific side chain Forssman antibodies might function as opsonins for bacteria with the 0 antigen determinant on their surface

In mutants of *E coli* 0 111 B4 extension of the LPS favours virulence resistance to phagocytosis and persistance in the peritoneal cavity (*Medearis et al* 1968) In our studies there is some correlation between

virulence and phagocytosis resistance, but the connection between these two parameters and chemotype is less strict As with virulence, the high resistance towards phagocytosis of MS, R4a and R9 seems to be dependent on the presence of B antigen on the surface (*Edebo* & *Normann* 1970, *Holme et al* 1969) In contrast, the presence of large numbers of 0-antigen determinants, probably as haptenic material, on the cell surface of R6 *(Edebo* & *Normann* 1970 *Holme et al* 1969) neither conveyed virulence (*Edebo* & *Normann* 1970), nor ability to evade phagocytes In general, however, several investigations into immunological mechanisms, as studied with LPS-mutants of enterobacteria, are consistent with the view that the 0 antigen determinant of the LPS, and to some extent the core sugars, are important virulence factors However, the topography of such substances or chemical groups of the bacterial surface should not be overlooked

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EXPERIMENTAL STUDIES ON CHOLERA IMMUNIZATION

1. *The Response of Neutralizing and Vibriocidal Antibodies in Rabbits after Immunization with Culture Filtrate Material from* V. cholerae

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The response of neutralizing and vibriocidal antibodies was studied in rabbits which were immunized with varied doses of a *V. cholerae* culture filtrate material (IA) containing exotoxin and endotoxin at a ratio of 1:100. Optimal formation of neutralizing as well as vibriocidal antibodies after a single subcutaneous injection of IA was noted when a dose of IA containing 12.5 µg exotoxin and 1.25 mg of endotoxin was employed. Irrespective of antigen dose the neutralizing antibodies were almost exclusively of the IgG class. Vibriocidal antibodies were of the IgG as well as IgM type, the IgG/IgM ratio increasing with rising antigen dose. Re[illegible]

Higher antibody titres than in the primary response were noted with low antigen doses. With high doses the titres were similar after the two injections but the maximum level was reached earlier than after the primary injection. In addition, after the secondary injection a higher proportion of IgG antibodies was noted.

Recent field trials in East Pakistan, West Bengal and the Philippines have shown that vaccination against cholera confers up to about 80 per cent protection to the vaccinated population, but the induced immunity is of short duration, lasting for only some 3-6 months (5, 13, 15). The employed commercial vaccines consist of formalin killed whole-cell vibrios, capable of inducing production of substantial amounts of vibriocidal antibodies but generally eliciting no neutralizing antibodies (6). However, cholera infection is known to give rise to formation of both types of antibodies (1). It seems reasonable that an immunizing agent leading to synthesis of neutralizing in addition to vibriocidal antibodies might give a more effective and durable immunity to cholera than the hitherto employed vaccines, since a protective role of neutralizing antibodies is indicated in recent animal experiments (4). Such an agent inducing both types of antibodies is easily obtained in the form of cell-free culture filtrate of *V. cholerae*, as demonstrated in a previous report (10).

In field trials it is difficult to investigate the optimal conditions for immunization due to the many different aspects of the immune response that should be considered. Experimental studies in animals are more apt to elucidate such important factors as the antibody response in relation to antigen dose, the immunoglobulin class of the antibodies

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formed and the possibility to elicit an anamnestic type of antibody response The aim of the present report was to study these factors concerning vibriocidal as well as neutralizing antibodies in rabbits immunized with a cholera vaccine consisting of culture filtrate material from *V cholerae* containing both exotoxin and endotoxin

MATERIALS AND METHODS

Immunizing agent A cell free culture filtrate of *V cholerae*, serotype Inaba obtained from the US National Institute of Health (lot 4493 G) was filtered through a pellicon membrane which eliminated low molecular non antigenic material (10) The fraction retained by the membrane was lyophilized and used as immunizing agent (IA) It contained four antigenic factors demonstrable in immunodiffusion analyses one of which consisted of exotoxin and another of endotoxin (10)

To estimate the content of exotoxin in IA the amount of IA needed to inhibit the neutralizing activity of a homologous antiserum was compared with the amount needed of purified choleragenoid (7) (i e a toxoid of the exotoxin) The content of endotoxin in IA was determined by comparing its capacity with that of a purified Inaba lipopolysaccharide to inhibit the vibriocidal activity of an anti Inaba serum It was found that on a dry weight basis these ratios were 1000 1 for IA/choleragenoid and 10 1 for IA/lipopolysaccharide i e the content of exo- and endotoxin in lyophilized IA preparation was about 0 1 per cent and 10 per cent respectively

Immunization schedule Eight 3 animal groups A H of four months old rabbits were used for immunization IA in the doses shown in Table 1 was administered subcutaneously in a volume of 1 ml without adjuvant Four months later an identical dose was given

Serum samples Bleedings were taken from the animals immediately before the first injection of IA and then at the intervals shown in Fig 3 Serum was dispensed into 0 5 ml aliquots and kept at −30°C until used Preliminary experiments with the samples taken 2 and 3 weeks after each injection showed that the antibody titres of the sera from the individual rabbits in each group did not differ more than one titre step For practical reasons, the presented results are therefore those obtained with pooled serum samples from the individual rabbits within each group

TABLE 1 *Dose of IA and Its Content of Endotoxin and Exotoxin Administered to the Animal Groups*

Animal group	Dose of IA (mg)	Endotoxin content (mg)	Exotoxin content (mg)
A	8×10^{4}	$8 \times 10^{-}$	8×10
B	4×10^{3}	4×10^{4}	4×10^{4}
C	2×10^{2}	2×10^{2}	2×10^{4}
D	1×10^{1}	$1 \times 10^{-}$	1×10^{4}
E	5×10^{1}	5×10^{3}	5×10^{4}
F	2 5	$2\ 5 \times 10^{1}$	$2\ 5 \times 10^{4}$
G	12 5	1 25	$1\ 25 \times 10$
H	62 5	6 25	$6\ 25 \times 10$

Gel filtration Gel filtration of antisera was performed through a 130 × 2 5 cm column of agarose (Biogel A5 m Bio rad Lab Richmond Ca) using for elution an 0 05 M phosphate buffer with 0 3 M sodium chloride pH 7 0 Flow rate was 16 ml/hr and the extinction of the eluate was continuously measured at 254 mμ Two ml of serum was filtered through the column and the eluate was pooled in

holm Sweden) the fractions were tested for immunoglobulin content by double diffusion and single radial immunodiffusion employing antisera specific for rabbit IgG IgA and IgM respectively (Miles Lab Inc Kankakee Ill and Nordic Til

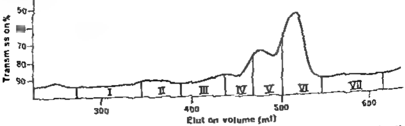


Fig 1 Typical agarose gel filtration pattern obtained with rabbit antisera fractions indicated by Roman numerals

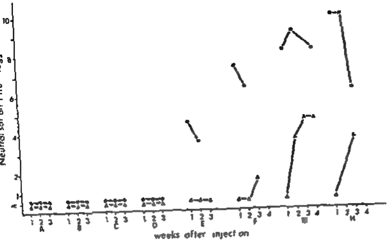


Fig 2a

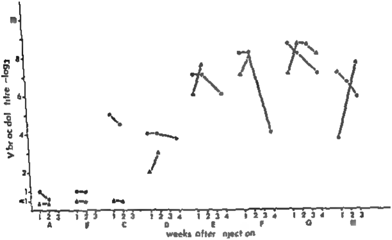


Fig 2b

Fig 2 Relation between immunization dose and titre of neutralizing (a) and vibriocidal antibodies (b) in animal groups A – H

▲ Titres after primary injection

● Titres after secondary injection four months later

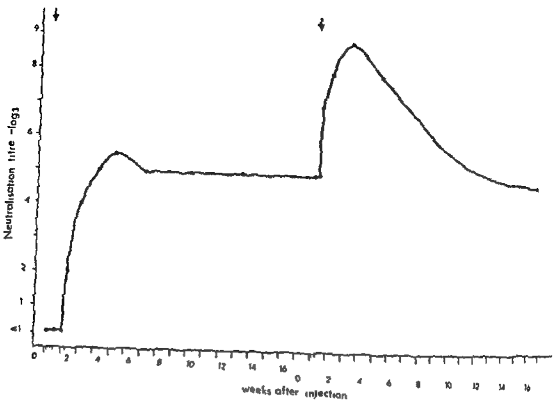
burg Netherlands) These analyses showed that fraction I only contained IgM, fraction III mainly IgA but low amounts also of IgG, and fraction V exclusively IgG

Neutralization tests Titration of neutralizing antibodies was performed with the intradermal test as described by *Benenson et al* (1)

Vibriocidal tests Vibriocidal antibody titrations were performed by means of the spot agar plaque technique earlier described (11)

RESULTS

Dose-response and anamnestic memory In a single subcutaneous injection only the three highest doses of IA (groups F-H) induced formation of detectable levels of neutralizing antibody In Fig 2a showing the dose-response relation it can be seen that in none of the responding groups F-H such antibodies were present one week after the injection but



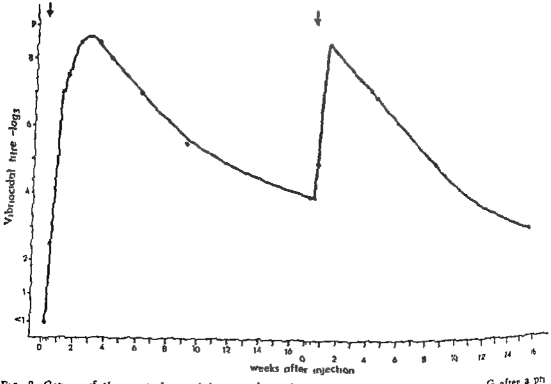


Fig 3 Course of the neutralizing (a) and vibriocidal (b) antibody responses in group G after a primary and a secondary injection of IA Arrow indicates time for injection

TABLE 2 *Distribution of Neutralizing and Vibriocidal Antibodies in the Agarose Gel Filtration Fractions*

Animal group	Weeks after primary (P) or secondary (S) injection		Antibody neutralizing (N) vibriocidal (V)	Titre –log			
				Whole serum	Gel filtration fractions IgM	IgA*	IgG
E	1	(P)	N	–	–	–	–
			V	9	9	2	6
	2	(P)	N	–	–	–	–
			V	12	7	–	6
	1	(S)	N	7	–	–	4
			V	11	6	2	8
	2	(S)	N	6	–	–	4
			V	11	6	2	[illegible]
F	1	(P)	N	–	–	–	–
			V	9	6	–	[illegible]
	2	(P)	N	–	–	–	–
			V	11	6	–	6
	1	(S)	N	11 5	–	–	7
			V	11	6	2	7 5
	2	(S)	N	10	–	–	6
			V	11	7	4	11
[illegible]	1	(P)	N		–		–
			V	11	6	6	6
	1 2/7	(P)	N	3	–	–	–
			V	12	8	2	9 5
	3	(P)	N	7	–	–	3
			V	13 5	7	1	9
	17	(P)	N	7	–		6
			V	8	1		4
	3/7	(S)	N	10 5		3	7
			V	8	4	3	4 5
	2	(S)	N	14	–	–	12
			V	9	6	3	11

* IgA contaminated with IgG

appeared in the 2 3 week samples Optimal *primary* response was noted in group G given *12 5 mg of 1A* while five fold lower or higher doses induced an *impaired* response Revaccination with identical *doses* four months after the first injection resulted in marked *increases of the titres* as compared to those seen in the primary response After the second injection high titres were present as early as one week after the reinjection, *now* also in group E which did *not respond to* the first injection (Fig 2a)

Vibriocidal antibody *formation* was observed after a single *injection of 1A* in the five highest doses *i e* in *groups D H* with maximal response in *group* G (Fig 2b) After the revaccination four months later vibriocidal antibody titres were noted in all groups. Of the groups showing a response after the first injection only group D responded with higher titres after the revaccination The maximum vibriocidal antibody level was however also in groups E H noted earlier after the revaccination than after the primary injection

Course of the antibody response The course of the antibody response was studied in greater detail in group G which responded with the highest neutralizing as well as vibriocidal antibody titres (Fig 3) Neutralizing antibody levels were first detected 9 days after the primary injection and rose ninefold

during the next 5 days to reach maximum titres after four weeks (Fig 3a) After a slight decrease the levels stayed constant for 10 weeks, i e to the time for the reinjection The reinjection with an identical dose induced a secondary type of neutralizing antibody response with a nine fold increase of titres noted within three days and with about 27 fold higher maximal levels present 2 weeks earlier than in the primary response A gradual titre decrease was noted during the next six weeks with approximately a halving of the titres every 8 days Thereafter the titres were nearly constant for at least another 3 months

As shown in Fig 3b vibriocidal antibodies were noted as early as three days after the first injection increased rapidly in titre and reached the maximum value after about 2 weeks After the peak value the titres decreased at an almost constant rate leading to a halving of the titres every 8 days Revaccination resulted in similarly high maximal antibody level as in the primary response but achieved about 2 weeks earlier This stage was followed by a decline at about the same rate as after the first injection

Immunoglobulin class Separated immunoglobulin fractions of sera were tested for contents of neutralizing and vibriocidal antibodies The results obtained with the IgM (I) IgG (V) and IgA (III) fractions of representative antisera from groups E G are shown in Table 2 Neutralizing antibodies were present almost exclusively in the IgG fraction even in the early primary response and increased in titre after revaccination and/or by rising doses of IA Neutralizing IgM antibodies were noted in only one sample taken early in the primary response from the animals of group H and present only in very low titre

Vibriocidal antibodies appeared in high titres in the IgM as well as in the IgG fractions but not in the IgA fraction (Table 2) A higher proportion of IgG to IgM antibodies was obtained by increasing the antigen dose as well as by revaccinating with the same dose

DISCUSSION

The agent used for immunization (IA) consisted of culture filtrate material from *V cholerae* containing about 10 per cent endotoxin and 0 1 per cent exotoxin (choleragen) This agent was chosen since it induces formation of vibriocidal anti endotoxin antibodies as well as neutralizing anti-exotoxin antibodies i e the two types of antibodies regarded to constitute a first and a second line of immune defence against cholera. In addition the content of endotoxin in IA could be expected to have an adjuvant effect on the neutralizing antibody response since bacterial endotoxin has been shown to play this role for other protein antigens (14)

The great dose range of IA employed in this study provides information which might be of interest for the planning of human vaccination trials with similarly composed vaccines The possibility to induce by a first injection an immunologic memory which could be utilized for eliciting a secondary type of response on revaccination was studied in some detail The results indicate that for the exotoxin constituent over a more than 100 fold varied dose range such a memory was induced (cf Fig 2a) For the endotoxin constituent the memory was found to be dose dependent With low doses an obvious secondary type of response with increased peak titres after the reinjection was noted while this was not the case with higher antigen doses (cf Fig 2b) It should be noted that also with the latter doses the peak values appeared earlier after the second than after the first injection and furthermore the IgG to IgM ratio increased considerably indicating a maturation of the response The low vibriocidal activity in some of the IgA fractions was probably caused entirely by contaminating IgG since IgA antibodies cannot activate the complement system through the classical pathway starting with C1 fixation (12) However aggregated IgA was recently shown to activate the complement at the C3 level (20) Accordingly it is conceivable that IgA antibodies may be able to

utilize late complement components and thereby participate in e g vibriocidal reactions

The relevance of studying the antibody response at the serum level after vaccination against a strictly intestinal disease like cholera is debatable, especially since there is considerable experimental evidence suggesting that locally formed copro antibodies are the most effective mediators of immunity against infection with cholera vibrios (2 9) However, the close association noted in the Pakistan field trials between cholera protection and serum titre of vibriocidal antibodies indicates that serum antibodies, if not protective in themselves, at least correlate well to the degree of immunity

Field trials are lacking concerning the relation between serum titre of neutralizing antibodies and cholera attack rate An inverse correlation between these parameters was, however, demonstrated in dogs on orogastric or Thiry-Vella loop challenge with exotoxin (4) Further perfusion of canine ileal segments with blood from 'pumper dogs' immunized with exotoxin protected the segments against the effects of exotoxin challenge, indicating that neutralizing antibodies in the serum are protective (3) The latter view was supported by experiments employing the rabbit ileal loop system, where it was found that subcutaneous immunization with IA as well as intravenous administration of serum from the vaccinated animals conferred significant neutralization (unpublished)

Based upon the results in the present study where practically all of the neutralizing capacity of serum resided in the IgG fraction it seems likely that the protective immunity against cholera exotoxin mediated by the blood and blood serum respectively, in the aforementioned reports was obtained by antibodies of the IgG class This assumption is in agreement with the finding of *Robbins* (17) that IgG antibodies were much more effective than IgA and IgM antibodies in neutralizing diphtheria toxin The low neutralizing capacity of antibodies of the latter two immunoglobulin classes could possibly explain why no IgM anti exotoxin antibodies were detected in the primary response If formed, the IgM antibodies are probably more likely to be detected by the passive haemagglutination method described by *Finkelstein* & *Peterson* (8), since agglutination techniques generally are more sensitive in revealing IgM antibodies (17)

It seems reasonable, as pointed out in the introduction that future cholera vaccines for parenteral use should have the capacity to induce neutralizing as well as vibriocidal antibodies i e contain exotoxin or -toxoid in addition to endotoxin Such an immunizing agent could consist of the conventional whole cell vaccine or purified endotoxin (19) supplemented by a purified exotoxin However the complicated procedures described for isolating exotoxin (7, 10, 16) will probably limit the employment of such a vaccine for common use An altenative vaccine of similar composition could be the easily obtained culture filtrate material, IA, employed in this study, possibly after formalin detoxification The animal experiments described in the present report suggest that this immunizing material containing exotoxin and endotoxin at a ratio of about 1 100 might also be suitable for inducing high levels of vibriocidal as well as neutralizing antibodies in humans

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We are grateful to Mrs *Gun Wallerstrom* for skilled technical assistance

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A NEW PNEUMOCOCCUS TYPE

Type 47 A

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A new pneumococcus type, 47 A has been established bringing the number of known types up to 83 The new type is closely related to type 47 (now called 47 F) and the two types form group 47 Type 47 A has an antigen in common with type 43

In 1967 a new pneumococcus type, 12 A, was published (*Lund & Munksgaard* 1967) bringing the number of known types up to 82 In May 1971 Statens Seruminstitut received four strains from Edinburgh, Scotland, for pneumococcus typing These proved to be identical and to be different from the known types The new type was designated type 47 A

The four strains were isolated from a patient with a chronic, purulent bronchitis He originally carried pneumococcus of group 23 at the time of an exacerbation and then over a longer period pneumococcus of group 10 During an exacerbation in April 1971, four optochin sensitive strains were isolated from sputum and pharynx These did not give satisfactory capsular reactions with any of the pneumococcal typing sera produced at Statens Seruminstitut, Copenhagen The strains were therefore sent for examination to the Pneumococcus Department of that institute Here the four strains gave identical reactions *viz* capsular reaction in diagnostic pooled serum G and I, and in type serum for type 43 (*Morch* 1944) and type 47 (*Lund* 1957) In repeated subcultures from single colonies, the strains proved to be pure cultures and not mixtures of types 43 and 47 The new type was found to be closely related to type 47 and to have a smaller antigen in common with type 43 Thus, it was convenient to place type 47 and the new type into one group, *viz* group 47, consisting of type 47 F = the original type 47, and type 47 A = the new type

Type 47 A (strain L 351) was found to be a gram positive diplococcus, being soluble in bile, without growth on 40 per cent bile agar, sensitive to optochin giving aerobic growth of smooth colonies on 5 or 10 per cent blood agar, showing α haemolysis and giving homogeneous growth in serum broth

Sensitivity The strain was tested in the Antibiotics Department of Statens Seruminstitut (Chief Dr *Jørgen Bang*) and was found to be fully sensitive to penicillin, sulphonamides, tetracyclines erythromycin, chloramphenicol, novobiocin methicillin, vancomycin, fucidin, lincomycin, ampicillin and cephalosporins, and resistant to streptomycin, kanamycin, polymyxins, gentamycin, and nalidixan

Virulence Type 47 A showed low virulence for mice, while type 47 F was found to be of moderate virulence

Biochemical reactions After 8 days at 37° C, type 47 A did not ferment arabinose, xylose, dulcitol, inositol and sorbitol, but gave positive reaction on the first day in galactose lactose, sucrose and maltose Glucose was fermented on the second day without gas Inulin was positive on the first

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day and salicin on the second, while mannitol was negative after incubation for 8 days (compare *Mørch-Lund* 1949)

Antipneumococcal serum Serum was produced from one of the original strains (L 351) by immunizing ten rabbits (*Lund* 1960) The strain gave capsular reaction and agglu-

TABLE 1 *Cross Reactions of Pneumococcus Type 47 A (strain L 351)*

Sera	Strain of type 47 A capsular titre	Strain of type 47 A agglutination titre
43	64	64
47 F	256	128

TABLE 2 *Cross Reactions of Different Batches of Pneumococcus Serum 47 A*

Serum 47 A Batch number	Date	Pneumococcus types 47 A		47 F		43	
		c	a	c	a	c	a
1	26-7-71	256	512	64	32	16	2
2	23-8-71	512	512	64	128	16	8
3	21-9 71	512	1000	128	128	4	4
4	19-10-71	256	512	128	64	8	8
5	16-11-71	512	1000	128	128	16	4

[illegible] = capsular titre, a = agglutination titre
Weak reactions (capsular titres in brackets)
Batch 1 37(8), 48(2)
Batch 2 11F(4), 11A(2), 35F(2), 35A(2), 37(4), 48(4)
Batch 3 48(2)
Batch 4 11F(2), 35F(4), 35A(2), 48(2)
Batch 5 11A(2), 35F(4), 35A(4), 48(4)

TABLE 3 *Cross Absorptions of Types 43, 47 F and 47 A*

Sera		*Serum titres with pneum strains* 43		47 F		47 A	
		c	a	c	a	[illegible]	a
43	not absorbed	128	256	0	0	64	64
	abs with 47 F	128	64	0	0	32	64
	abs with 47 A	64	64	0	0	[illegible]	0
47 F	not absorbed	0	0	512	512	256	128
	abs with 43	0	0	256	256	128	128
	abs with 47 A	0	0	64	128	0	0
47 A	not absorbed	16	2	64	32	256	512
	abs with 43	0	0	64	64	256	512
	abs with 47 F	4	2	0	0	256	512

c = capsular titre, a – agglutination titre
Antigenic formulas
Pn 43 = 43a, 43b
Pn 47 F = 47a, 35a 35b
Pn 47 A = 47a 43b

TABLE 4 *Diagnostic Pneumococcus Sera Reacting with Type 43 and Group 47*

Diagnostic sera	Capsular titres with strains 43	47 F	47 A
Type 43	128	0	64
Group 47	0	64	128
Pool G	0	64	[illegible]
Pool I	32	0	16
Omni	16	32	4
Factor 43b	4	0	256

TABLE 5 *Capsular Reactions of Type 43 and Group 47 in Diagnostic Sera*

Strains	Diagnostic sera Pool G	Pool I	Type 43	Group 47
43	—	+	+	—
47 F	+	—	—	+
47 A	+	+	+	+

TABLE 6 *Danish Type Designations of 83 Pneumococci Antigenic Formulas According to Kauffmann & Lund (Changed by Lund 1970)*

Type	Antigenic formula	Type	Antigenic formula
1	1a	20	20a 20b 7g
2	2a	21	21a
3	3a	22F	22a 22b
4	4a	22A	22a 22c
5	5a	23F	23a 23b 18b
6A	6a 6b	23A	23a 23c 15c
6B	6a 6c	23B	23a 23b 23d
7F	7a 7b	24F	24a 24b 24d 7h
7A	7a 7b 7c	24A	24a 24c 24d
7B	7a 7d 7e 7h	24B	24a 24b 24e 7h
7C	7a 7d 7f 7g 7h	25	25a 25b
8	8a	27	27a 27b
9A	9a 9c 9d	28F	28a 28b 16b 23d
9L	9a 9b 9c 9f	28A	28a 28c 23d
9N	9a 9b 9e	29	29a 29b 13b
9V	9a 9c 9d 9g	31	31a 20b
10F	10a 10b	32F	32a 27b
10A	10a 10c 10d	32A	32a 32b 27b
11F	11a 11b 11c 11g	33F	33a 33b 33d
11A	11a 11c 11d 11e	33A	33a 33b 33d 20b
11B	11a 11b 11f 11g	33B	33a 33c 33d 33f
11C	11a 11b 11c 11d 11f	33C	33a 33c 33e
12F	12a 12b 12d	34	34a 34b
12A	12a 12c 12d	35F	35a 35b 34b
13	13a 13b	35A	35a 35c 20b
14	14a	35B	35a 35c 29b
15F	15a 15b 15c 15f	35C	35a 35c 20b 42a
15A	15a 15c 15d 15g	36	36a 9e
15B	15a 15b 15d 15e 15h	37	37a
15C	15a, 15d 15e	38	38a 25b
16	16a 16b 11d	39	39a 10d
17F	17a 17b	40	40a 7g 7h
17A	17a 17c	41F	41a 41b
18F	18a 18b 18c 18f	41A	41a
18A	18a 18b 18d	42	42a 20b 35c
18B	18a 18b 18e 18g	43	43a, 43b
18C	18a 18b 18c 18e	44	44a 44b 12b 12d
19F	19a 19b 19d	45	45a
19A	19a 19c 19d	46	46a 12c 44b
19B	19a 19c, 19e 7h	47F	47a, 35a 35b
19C	19a 19c 19f 7h	47A	47a 43b
		48	48a

tination in unabsorbed sera of pneumococcus types 43 and 47 F, but not in sera of the remaining 80 types (Table 1) Heart puncture was made every four weeks

The different batches were examined for cross reactions with all known pneumococcus types The reactions with types 43 and 47 F were present in all sera and furthermore in all batches there were weak cross reactions with other types (Table 2)

Cross absorption Cross absorption tests between types 43, 47 F and 47 A showed that type 47 A was so closely related to type 47 F that it was practicable to put both types into one group 47 (Table 3)

On the basis of the cross absorption tests the antigenic formula for type 47 A can be stated as 47 a, 43 b Type 43, the formula of which was 43 a is now given an additional antigen, 43 b, in common with type 47 A Thus, the antigenic formulas for the three types are

Type 43 = 43 a 43 b
Type 47 F = 47 a 35 a, 35 b
Type 47 A = 47 a, 43 b

Diagnostic sera The third heart puncture was chosen for production of diagnostic serum for group 47 (type 47 F + type 47 A) This serum (Table 2) has only weak heterologous reactions with types 43 (capsular titre 4) and 48 (capsular titre 2) The homologous capsular titres are for type 47 F 128 and for type 47 A 512 After absorption of the heterologous reactions a diagnostic serum specific for group 47 is obtained, with capsular titres for type 47 F 64 and type 47 A 128

Type 47 A gives capsular reaction in the diagnostic sera used at present for pools G and I, in the polyvalent 'omni serum, in type serum 43 and in group serum 47 (Tables 4 and 5)

The reactions of type 47 A in pool I and serum 43 could be removed by absorption but in that way the titres for type 43 would be reduced too much for practical use It has therefore been decided not to remove these cross reactions If capsular reactions are found both in pool G and I and in serum 43 and 47, the strain must be of the new type 47 A

In order to differentiate the types in group 47, a 'factor' serum 43 b may be used This is produced by absorbing serum 47 A with vaccine of type 47 F (Table 3) The Danish type designations of 83 pneumococcus types are shown in Table 6

The authors are grateful to Dr *M E Anderson* who provided the case history of the patient and to Mrs *M G G Malcolm* and Mrs *K C Pratt* for technical assistance

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RABBIT ANTI RAT LYMPHOCYTE SERUM: IMMUNOSUPPRESSION MEDIATED BY IgM ANTIBODIES

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Rabbit anti rat lymphocyte sera (ALS) were produced in rabbits treated with azathioprine during the immunization period Such antisera were cytotoxic to lymphocytes with nearly as high titres as have been seen in untreated animals using the same immunization schedule Treatment of the antisera with 2 mercaptoethanol followed by alkylation resulted in marked loss in titre towards lymphocytes Separation on Sephadex G 200 confirmed that most of the cytotoxic activity was to be found among the IgM antibodies Both "thymus-azathioprine and spleen azathioprine ALS significantly prolonged the survival of skin allografts in rats Thymus antisera were more efficient than spleen antisera Reduction and alkylation of active thymus antisera resulted in loss of in vivo immunosuppressive effect

The immunosuppressive activity of antilymphocyte sera (ALS) has been shown to reside in the IgG fraction of some antisera (1, 2, 4, 7) *Mandel & Asofsky* found, however, that the IgM as well as the IgG fraction contained immunosuppressive activity (10) In previous reports (5 6) lymphocytotoxic activity was demonstrated both in the IgM and the IgG fraction of rabbit anti rat lymphocyte sera Antisera with the greatest ability to delay the rejection of skin allografts contained most in vitro cytotoxic activity in the IgM fraction

In an attempt to produce ALS with in vitro lymphocytotoxic activity mostly in the IgM fraction rabbits were treated with azathioprine during the immunization period Azathioprine has been shown to modify the immune response by suppressing IgG antibody formation (12, 13) The present paper describes the behaviour in vitro and the immunosuppressive activity of the antisera obtained

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MATERIALS AND METHODS

Preparation of ALS

Animals Albino rabbits used for raising of antisera weighed between 2 5 and 3 5 kg at the time of immunization They were of both sexes and were fed commercial rabbit diet

Preparation of antigen Rat spleens or rat thymuses were cut into fragments and gently

(Ljungberg Stockholm Sweden) using a cetrimide solution (9) The relative number of viable cells was determined using freshly prepared 0 2 per cent Trypan Blue in Hanks solution Viability counts consistently showed 60–80 per cent viable cells The cell suspensions were used for immunization within one hour of preparation

Immunization procedure Two groups of rabbits were immunized one using rat spleen cells the other using rat thymus cells Both groups were immunized three times at weekly intervals The animals were given 0 8 1 4 × 10⁹ viable cells intravenously *All* animals were given daily subcutanous injections of azathioprine 6 mg/kg body weight into the loose tissues of the neck On average, each rabbit received 15 mg azathioprine daily from the day of the first immunization to 7 days following the final immunization i e for a total of 22 days Ten days after the last immunization the rabbits were bled by heart puncture under Nembutal® anaesthesia Serum was collected as previously described (5)

Samples of antisera were kept for in vitro studies The rest of the serum was pooled according to the immunization procedure thus obtaining two pools of antisera referred to as spleen azathioprine ALS and thymus azathioprine ALS Each batch of pooled serum was dialysed 3 times against 50 volumes of phosphate buffered 0 15 M NaCl, pH 7 2 for 24 hours in order to remove active metabolites of azathioprine The antisera were then stored at —20° C until used

Absorption of anti red cell activity After inactivation by heating to 56° C for 30 min, antisera were absorbed with rat red blood cells, washed thrice in 0 15 M NaCl Repeated absorptions were carried out until the haemagglutination titre was less than 16 using a 1 per cent solution of washed rat red blood cells in 0 15 M NaCl The antisera were then sterilized by passage through a 0 45 μ Millipore filter

In vitro studies

The cytotoxic test The technique for the preparation of the lymphocyte suspension and the procedure of the cytotoxic test have been described previously (5)

Mercaptoethanol (ME) treatment This was carried out as described previously using a final molarity of 0 1 M 2 mercaptoethanol for 2 hours at room temperature followed by alkylation with 0 02 M iodoacetamide in a 0 06 M phosphate buffer pH 7 2 (5)

Gel filtration chromatography This was carried out as described before using Sephadex G–200 and a 0 05 M phosphate buffer Optical density was read in a Beckman DB spectrophotometer at 280 mμ Peak fractions were pooled as described previously designated pool I the IgM fraction or pool II the IgG fraction The pools were of equal volume being 30–35 ml The pools were not concentrated before testing for in vitro activity (5)

In Vivo Studies

Administration schedule Sera were given subcutanously according to the following schedule 2 ml daily from day – 7 to day –1 1 ml daily from day 0 (the day of grafting) to day + 7 Each rat thus received a total of 22 ml absorbed and filter sterilized serum

Skin grafting technique Full thickness skin grafts were transplanted from inbred Fischer male rats to inbred hooded BDE male rats as previously described (6) 5 groups of rats were compared untreated controls (10 animals) rats treated with normal rabbit serum (5 animals) spleen azathioprine ALS (10 animals) thymus azathioprine" ALS (10 animals) and ME treated thymus azathioprine ALS (5 animals)

RESULTS

In Vitro Lymphocytotoxic studies

The results of in vitro test for lymphotoxicity are given in Table 1 All crude antisera were cytotoxic to lymphocytes There was some variation between the individual antisera A marked decrease of lymphocytotoxic activity was observed in all antisera treated with ME followed by alkylation with iodoacetamide This fall in titre from 512 to 32 for the spleen azathioprine ALS and from 256 to 8 for the ' thymus-azathioprine ALS indicates that most of the lymphocytotoxic antibodies were sensitive to ME and thus possibly of IgM nature Separation of crude antiserum on Sephadex G 200 confirmed that there was more lymphocytotoxic activity in pool I the IgM fraction than in pool II, the IgG fraction of most of the individual antisera The pools of absorbed and sterile antisera showed the same pattern

Skin grafting Survival

No wasting or haematuria was observed in any of the rats treated with absorbed antisera

Untreated hooded BDE rats rejected Fischer skin grafts between the 11th and the 13th day Only 10 per cent showed viability on day + 12 Normal rabbit serum did not prolong skin graft survival (Table 2 Fig 1)

Compared to the controls both the spleen azathioprine ALS and the thymus azathioprine ALS showed statistically significant prolongation of skin allograft survival There was however a marked difference between these two types of antisera Treatment with

TABLE 1 *In Vitro Lymphocytotoxic Titres of Individual and Pooled Different Rabbit Anti Rat Lymphocyte Sera Comparison of Activity of Crude Antiserum, Sterile Absorbed Serum, Mercaptoethanol Treated Serum and of IgM and IgG Fractions*

Type of serum		Crude serum	Sterile absorbed serum	ME-treated serum	IgM fraction	IgG fraction
"Spleen azathioprine ALS		1024		16	16	4
		512		32	16	4
		512		32	4	4
	pooled	512	512	32	8	4
Thymus azathioprine ALS		1024		16	16	4
		256		8	4	<4
		512		16	16	4
	pooled	256	256	16	8	<4
"Thymus azathioprine ALS treated with mercaptoethanol and alkylated with iodoacetamide			16	16	<4	<4

TABLE 2 *Comparison of in Vitro Lymphocytotoxic Titres and Effect on Skin Allograft Survival (Fischer to Hooded BDE) of Normal Rabbit Serum and Three Different Types of Rabbit Anti Rat Lymphocyte Sera Raised Using Spleen Cells as Antigen*

Type of serum	In vitro lymphocytotoxic titre				Skin allograft survival (mean ± S D)
	Crude serum	ME-treated serum	Sephadex G-200		
			IgM fraction	IgG fraction	
Normal rabbit serum*	16	4	4	4	10 0±0
"3 pulse ALS*	2048	128	16	8	21 1±0 77
8 pulse ALS*	2048	256	8	32	16 0±1 48
Spleen azathioprine ALS	512	32	8	4	15 5±0 53

* from *Jakobsen* (6)

the "thymus azathioprine ALS resulted in an average skin graft survival of 19 1 days while similar treatment with the spleen azathioprine ALS resulted in an average survival of 15 5 days This difference is statistically significant ($p<0\ 001$)

Table 2 shows a comparison between the spleen azathioprine ALS of this study with other spleen antisera from a previous study 6) The reduction in cytotoxic activity after treatment with ME was more pronounced in the spleen azathioprine ALS than in the 3 pulse ALS

Crude thymus azathioprine ALS increased skin allograft survival to 19 1 days ($p<0\ 001$ versus controls) (Table 3, Fig 1) A batch of such antisera was treated with ME and alkylated with iodoacetamide This resulted in a drop of the cytotoxic titre from 256 to 16 (Table 1) A second reduction and alkylation did not reduce the titre further After separation on Sephadex G 200 the pooled fraction I containing the IgM antibodies had a titre of 8 while that of the ME-treated ALS had a titre of less than 4 Pooled fraction II containing the IgG antibodies did not show cytotoxic activity before nor after reduction Reduction had thus resulted in a drop of cytotoxic activity of the crude antisera by 4 steps and by abolishment of activity found in the IgM fraction This reduced batch did not show any ability to delay

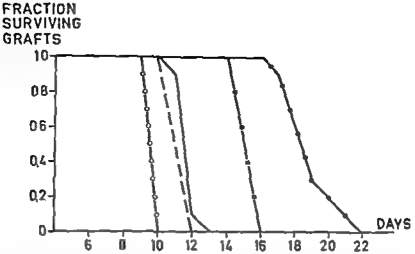

Fig 1 Survival curves for skin allografts from Fischer to hooded BDE rats Comparison of effect of different types of ALS ALS totalling 22 ml per rat given subcutaneously from day —7 to day +7 ——— untreated, —o—o— normal rabbit serum —■—■— 'spleen azathioprine ALS, —●—●— "thymus azathioprine" ALS, — — — ME treated 'thymus azathioprine ALS

TABLE 3 *Survival Data for Skin Allografts from Fischer to Hooded BDE Rats, Comparison of Effect of Two Different Types of Rabbit Anti Rat Lymphocyte Serum, and Mercaptoethanol Treated Antiserum*

Type of treatment	No of animals	Range	Mean ± S D	Student T test
None	10	11–13	12 0±0 45	
Normal rabbit serum	5	10	10 0±0	
'Spleen azathioprine' ALS	10	15–16	15 5±0 53	p<0 001 versus control
Thymus azathioprine' ALS	10	17 21	19 1±1 52	p<0 001 versus control p<0 001 versus spleen
Thymus azathioprine' ALS treated with mercaptoethanol and alkylated	5	11 12	11 6±0 55	p–0 1 versus control

the rejection of skin allografts Average skin graft survival was 11 5 days (p=0 1 versus controls) (Table 3, Fig 1)

DISCUSSION

Many investigators have demonstrated immunosuppressive activity of the IgG fraction of ALS (1, 2, 4, 7 10) *James & Medawar* (7), found no effect of their 19 S fraction, but crude antisera, raised in horses, lost in vivo effect after treatment with ME This suggested the existence of a group of IgG antibodies sensitive to ME (8) *Mandel & Asofsky* (10) have however, demonstrated an effect of the IgM fraction *against* the graft versus host reaction They also demonstrated that the in vivo effect of the IgM fraction was lost after treatment with ME They did not study the effect of reduced and alkylated crude antiserum *Brent et al* (3) showed that ME treatment did *not* affect the

in vitro activity of their 7 S fraction, but resulted in loss of in vitro activity of their 19 S fraction Neither of these fractions were, however, tested for immunosuppression In previous experiments (6) it was shown that antisera raised by short immunization schedule had the most potent immunosuppressive effect It was found that short pulse ('3 pulse') antisera also contained more IgM cytotoxic activity than did antisera raised with more prolonged immunization ('8 pulse') This suggested that some immunosuppressive activity might reside in the IgM fraction, and also indicated that the relative importance of the IgM and the IgG fractions might vary in individual antisera The comparison between different antisera, as regards immunosuppressive effect of IgM and IgG antibodies, cannot be done without paying attention to the immunization schedule

The present investigation was undertaken in order to raise antisera containing relatively more IgM cytotoxic antibodies than the sera studied previously The 3 pulse' schedule was chosen and in addition the antibody producing animals were given azathioprine treatment Azathioprine may abolish the immune response when given in large doses simultanously with the antigen (13) Animals given a sub inhibitory dose of 6 mercapto purine produce only small amounts of IgG antibodies but normal amounts of IgM antibodies (12) The in vitro behaviour of both spleen azathioprine and "thymus azathioprine' ALS confirmed that azathioprine treatment modifies the immune response The cytotoxic activity both of crude and sterile, absorbed sera was less than of previous "3 pulse" ALS (Table 2) Furthermore, treatment with ME followed by alkylation and by chromatography on Sephadex G 200 showed that the activity in both sera resided mostly in the IgM fraction Comparison between the present 'spleen azathioprine' ALS and the previous [redacted] pulse ALS make this difference clearer the titres after ME treatment was 32 and 256 respectively The IgM/IgG ratio as judged by in vitro activity corresponded being 8/4 and 8/32

The present study showed that both "spleen azathioprine" ALS and "thymus-azathioprine' ALS have immunosuppressive effect The cytotoxic activity of both antisera was markedly reduced after treatment with ME Furthermore, the "thymus azathioprine" ALS lost its in vivo immunosuppressive effect after reduction and alkylation These results strongly suggest that both antisera contained IgM antibodies with immunosuppressive activity There was statistically significant shorter skin allograft survival with the "spleen azathioprine' ALS versus the spleen "3 pulse' ALS (Table 2) The in vitro activity of the 'spleen azathioprine ALS was however, less than that of the spleen "3 pulse' ALS, and this may account for the difference in in vivo immunosuppressive effect

Thymus azathioprine' ALS was raised because thymus is thought to be a better antigen than spleen (4, 11) This proved to be the case, the difference in immunosuppressive effect between thymus and spleen ALS was statistically significant ($p<0.001$) With the good immunosuppressive effect of the "thymus azathioprine' ALS it was possible to demonstrate clearly the loss of effect after ME-treatment It may be argued that the reduction and alkylation of the "thymus azathioprine ALS of this study had resulted in 'inactivation' of immunosuppressive IgG antibodies Such treatment is known to affect the complement binding activity of IgG antibodies (5 14) The present experiments show however, that the reduced antisera did have some residual cytotoxic activity, and thus still had the ability to fix complement

Azathioprine treatment was discontinued 3 days before bleeding the serum raising animals, and all antisera were dialysed against large volumes of buffered saline in order to remove dialysable metabolites No technique is available to prove that this was obtained It must, however, be considered if traces of azathioprine or active metabolites might be responsible for the observed immunosuppressive effect. The significant different immunosuppressive effect of the thymus azathioprine and 'spleen azathioprine ALS would

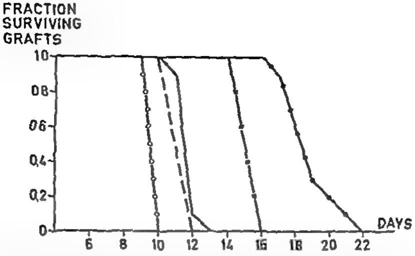

Fig 1 Survival curves for skin allografts from Fischer to hooded BDE rats Comparison of effect of different types of ALS ALS totalling 22 ml per rat given subcutaneously from day —7 to day +7 ——— untreated —o—o— normal rabbit serum —■—■— spleen azathioprine ALS —●—●— thymus azathioprine ALS — — — ME treated thymus azathioprine ALS

TABLE 3 *Survival Data for Skin Allografts from Fischer to Hooded BDE Rats Comparison of Effect of Two Different Types of Rabbit Anti Rat Lymphocyte Serum and Mercaptoethanol Treated Antiserum*

Type of treatment	No of animals	Range	Mean ± S D	Student T test
None	10	11–13	12 0±0 45	
Normal rabbit serum	5	10	10 0±0	
Spleen azathioprine ALS	10	15–16	15 5±0 53	p<0 001 versus control
Thymus azathioprine ALS	10	17 21	19 1±1 52	p<0 001 versus control p<0 001 versus "spleen"
Thymus azathioprine ALS treated with mercaptoethanol *and alkylated*	5	11 12	11 6±0 55	p 0 1 versus control

the rejection of skin allografts Average skin graft survival was 11 5 days (*p*=0 1 versus controls) (Table 3 Fig 1)

DISCUSSION

Many investigators have demonstrated immunosuppressive activity of the IgG fraction of ALS (1, 2 4 7 10) *James & Medawar* (7), found no effect of their 19 S fraction but crude antisera raised in horses, lost in vivo effect after treatment with ME This suggested the existence of a group of IgG antibodies sensitive to ME (8) *Mandel & Asofsky* (10) have however demonstrated an effect of the IgM fraction against the graft versus host reaction They also demonstrated that the *in vivo* effect of the IgM fraction was lost after treatment with ME They did not study the effect of reduced and alkylated crude antiserum *Brent et al* (3) showed that ME treatment did not affect the

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EVALUATION OF DISINFECTANT INACTIVATORS

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A procedure for the control of disinfectant inactivators is described and suitable antagonists for some disinfectants are listed.

Disinfectant testing is a complex and largely controversial subject. However, ever since *Koch* in 1881 assayed the efficacy of phenol in the killing of anthrax spores, have steps been included to rid the systems of superfluous disinfectant before subcultivation. This becomes necessary if bactericidal and not only bacteriostatic effect is to be measured. *Koch* washed his anthrax infected silk threads before being subcultured. Subsequently numerous reports on disinfectant testing have appeared, many apparently without using any disinfectant antagonistic step. In many instances the inactivators have evidently not been controlled.

In the following a procedure for the control of efficacy of inactivators is described.

MATERIALS AND METHODS

Test organism has been a strain of *Escherichia coli*.

Disinfectant preparations have been Aktivex®, Cidex®, Fenylfenolatsåpe®, Jodopax®, Kloramin T®, Resiguard®, Savlon®, Tafosan®, and Vesphene®*). The details of these have been described elsewhere (3).

Reagents of particular importance have been lecithin, type IIs from Sigma Chemicals, St. Louis, Mo., Tween 80 (polyethylene sorbitan monooleate), Lubrol W from Imperial Chemical Industries, Great Britain, sodium thiosulphate *pro analysi*, and sodium bisulphite, *pro analysi*.

Bacteriological procedures. The test strain was grown in 30 ml Nutrient Broth No. 2 (Oxoid) in 100 ml Erlenmeyer flasks which were stoppered with cotton plugs and contained 3 glass beads. The culture was kept overnight in a gyratory shaker at 37° C. The optical density of the culture was adjusted to correspond to 10^9 viable *E. coli* of an overnight culture per ml (Beckman Colorimeter Model D with green filter of maximum transmittance 510–580 nm).

Three parallels were prepared:

i. Inactivator + disinfectant made up in sterile tap water
ii. Inactivator in same concentration as above
iii. Sterile tap water

To 9 ml of each parallel was added 1 ml of a ca. 10^6/ml suspension of *E. coli*. The mixture was shaken briefly 10 times when samples of 0.1 ml were withdrawn for pour plate counts with 15 ml Nutrient Broth No. [illegible] (Oxoid) + 1.5 per cent Agar (Oxoid) of temperature 45° C. The time allowed for the inactivation step was kept short for two reasons. First of all, a sufficiently efficient inactivator allows measurements after short periods of disinfectant action. Secondly, this was suitable for a quantitative procedure employed subsequently (4, 5). All counts were made in triplicate.

Evaluation of test. An inactivator was accepted as adequate if the inactivator (parallel (ii) above) was not antibacterial in itself and the counts in the parallels (i) and (iii) were not significantly dif-

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*) The active ingredients of the preparations are shown in Table 4.

TABLE 1 *Example of Inactivator Test Protocol with Two Disinfectants*

Inactivator dilution	Plate count after observation periods in minutes*)		
	1	30	60
Resiguard®, 0 1 %			
7 % Lubrol W + 1 % lecithin	47**)	44	57
7 % Lubrol W + 2 % Tween 80	41	47	43
5 % Lubrol W + 1 % lecithin	6	0	0
5 % Lubrol W + 2 % Tween 80	0	0	0
Control without inactivator or disinfectant	61	52	54
Ivisol® 0 5 %			
7 % Lubrol W + 1 % lecithin + 2 % Tween 80	10	[illegible]	0
7 % Lubrol W + 1 % lecithin + 4 % Tween 80	30	32	28
Control without inactivator or disinfectant	37	ND	ND

ND = not done The lack of antibacterial effect of the inactivator alone was checked separately

*) Aliquots withdrawn after having been shaken 10 times

**) The figures represent the plate of a 10^{-5} dilution of the reaction mixture

TABLE 2 *Examples of Inactivator Modifications Necessary to Neutralize Increasing Disinfectant Concentrations*

Disinfectant	Dilution*) (%)	Inactivator
Aktivex®	0 5	3 % Lubrol W + 2 % lecithin
	1 2	3 % Lubrol W + 2 % lecithin
	2 0	3 % Lubrol W + 2 % lecithin
Talosan®	0 5	2 % Tween 80
	1 0	4 % Tween 80
	2 0	6 % Tween 80
Vesphene®	0 5	2 % Tween 80 + 2 % Lubrol W
	1 0	2 % Tween 80 + 2 % Lubrol W
	2 0	4 % Tween 80 + 4 % Lubrol W

*) The commercial product as is is considered 100 per cent

ferent The difference between counts was evaluated by a set of tables prepared by *Loren* which for observations adhering to a Poisson distribution show when figures are significantly different at the α = 0 05 level (*10*)

RESULTS

An example of a protocol for an inactivator test is shown in Table 1 In the example are shown the results of counting also after 30 and 60 minutes disinfectant inactivation These figures show that the initial event is decisive only non significant change in the mean plate counts appearing subsequently When the disinfectant concentration is increased there must be modifications in some of the disinfectant antagonists (Table 2)

The inactivator recommended in the literature for glutaraldehyde (*11*) sodium bisulphite turned out to be markedly antibacterial in concentrations down to 0 5 per cent A 1 per cent dilution is recommended (*11*)

For Savlon no satisfactory inactivator was found Table 3 shows the alternatives tested None of these alternatives were antibacterial

The inactivators which evolved from the tests are shown in Table 4

TABLE 3 *Mixtures Tested for Potential Antagonistic Effect to 0.5 per cent Savlon®. None Were Found Adequately Antagonistic to Be Used as an Inactivator in Disinfectant Testing*

3 % Lubrol W + 2 % lecithin
6 % Lubrol W + 4 % lecithin
0.18 % lecithin + 1.25 % Tween 80
7 % Lubrol W + 1 % lecithin + 2 % Tween 80
7 % Lubrol W + 2 % lecithin + 4 % Tween 80
4 % Tween 80 + 2 % lecithin
250 g $CaCl_2$/liter + 8 % Tween 80 + 4 % lecithin
250 g $CaCl_2$/liter + 10 % Tween 80 + 5 % lecithin

DISCUSSION

The necessity of including an inactivator step in the examination of disinfectants should be obvious and yet often no mention is made of this. The elaborate test battery of the Deutsche Gesellschaft fur Hygiene und Mikrobiologie (*1*) with its detailed methodological descriptions makes no allusion to disinfectant antagonists. A more recently introduced procedure such as the Capacity Test (3, 9) contains no provisions for specific inactivator steps. The same objection applies to the descriptions for phenol coefficients (6, 7). On the other hand, the British Standard (8) for quantitative assessment of quaternary compounds recommends 3 per cent Lubrol W + 2 per cent lecithin. The Use Dilution Method of the Association of Official Agricultural Chemists (AOAC) washes the carriers before subcultivation.

It is customarily presumed that phenolics are adequately inactivated by sheer dilution due to their high dilution coefficients. It is difficult, however, to be certain that the newer compounds in this group cannot interfere with bacterial growth even in lower concentrations. Indeed *Stedman et al.* (13) have demonstrated that carry over is significant also for phenolics.

In principle, disinfectant antagonists may be included in the growth medium used for the registration of survivors. However, unless virtually instant inactivation takes place, a protracted growth inhibitory effect is possible. Under such circumstances, it is also difficult to measure with certainty only short disinfection periods. In the present context, the inactivation procedure was designed to be compatible with the quantitative procedure for disinfectant testing described by British Standard 3260: 1960 (8). There, the disinfectant inactivation precedes quantitative plating of survivors.

For the disinfectants Cidex and Savlon, no suitable inactivators were found. The sodium bisulphite which has been used traditionally for the inactivation of 2 per cent glutaraldehyde (Cidex) turned out to have a con-

TABLE 4 *Inactivators Against Commercial Disinfectants*

Commercial product	Active ingredients	Maximum concentration cleared (%)*	Inactivator solutions
Aktivex	Cetylpyridinium chloride	2	3 % Lubrol W + 2 % lecithin
Cidex	Glutaraldehyde		None
Fenylfenolatsåpe	*o*-Phenylphenol	0.5	2 % Tween 80 + 1 % lecithin
[illegible]nol	Arylated and alkylated phenolics	0.5	7 % Lubrol W + 2 % lecithin + 4 % Tween 80
Kloramin T	Chloramine T	0.5	0.5 % sodium thiosulphite
Resiguard	Picloxidine + benzalkonium	0.1	7 % Lubrol W + 1 % lecithin
Savlon	Chlorhexidine + cetrimide		None
Talosan	*p*-Chloro-*m*-cresole	2	6 % Tween 80
Vesphene	*o*-Benzyl-*p*-chlorophenol + *o*-Phenylphenol	2	4 % Tween 80 + 4 % Lubrol W

* The commercial product is considered 100 per cent.

siderable antibacterial effect at concentrations down to 0 5 per cent, in spite of the fact that 1 per cent is recommended usage (11) A glutaraldehyde concentration of 0 5 per cent was employed in a capacity test in which the parallels with the would be-inactivator were more effectively killing bacteria than the glutaraldehyde alone (*unpublished results*) Tests on glutaraldehyde with the AOAC Use-Dilution Test (12) have not encountered the difficulty with sodium bisulphite since this procedure uses infected carriers which are rinsed before subcultivation

The inactivators tried out for Savlon had some but insufficient inactivation The producer had found Lubrol W to be satisfactory in his technique (*techn cal literature, ICI*)

In instances where no satisfactory disinfectant antagonist is found, the problem may be circumvented by the use of membrane filters and washing After washing, the filters may be applied directly on agar media for the observation of survivors For such use, filters with hydrophobic edge should be used to avoid trapping of disinfectants at the filter holder junctions The filter technique, however, has the disadvantage that it is less precise for quantitative purposes since, for technical reasons, it is not quite as easy to maintain an accurately fixed period of disinfection with this method

The method described enables the detection of even low grade antibacterial effects of the inactivators and allows a graded response for the disinfectant antagonism In principle, a sensitive organism should be used to detect also small remaining amounts of active disinfectants The inactivators should buffer the maximum load of disinfectant employed in a test

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TRANSFERABLE AND NON-TRANSFERABLE DRUG RESISTANCE IN ENTERIC BACTERIA ISOLATED FROM URINARY SPECIMENS IN NORTHERN SWEDEN

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Drug resistance was studied in Gram negative bacteria isolated from urine specimens About 2/3 of the strains were resistant to one or more of the antibiotics under study (ampicillin chloramphenicol nalidixic acid streptomycin sulphonamide and tetracycline) *Escherichia coli* and *Proteus* dominated among the bacteria isolated Multiple resistance was common tetracycline resistance being the most common resistance trait Out of 286 drug resistant strains 26 per cent were found to carry R factors that could be transferred to the *E coli* K 12 strain used as recipient Also among the R factors tetracycline resistance was the most common resistance R factors carrying as much as 5 resistance traits were found The frequency of R factors among patients from different wards and in different age groups were found to be quite similar

Japanese workers first reported that resistance to several common antibacterial drugs can be transferred simultaneously from one bacterium to another by cell to cell contact This is due to the transfer of extrachromosomal resistance determinants so called R (resistance) factors The epidemiology and genetics of R factors was first reviewed by *Watanabe* (22) and later by *Mitsuhashi* (14) *Meynell et al* (13) and by *Anderson* (2)

R factors have been found to be widespread among Gram negative bacteria from human beings as well as from livestock (15 16, 21) and laboratory animals (20) R factors seem to be prevalent in many parts of the world (7) and their clinical importance has been clearly demonstrated during outbreaks of *Salmonella* and *Shigella* infections (2 14 22)

Infection of the urinary tract is the most common clinical problem created by enteric bacteria This is a report of the occurrence of R factors in Gram negative bacteria isolated from urinary specimens obtained at the bacteriological laboratory of Umeå in Northern Sweden

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Requests for reprints should be addressed to Dr *Kurt Nordstrom* Department of Microbiology University of Umeå S 901 87 Umea 6 Sweden

MATERIALS AND METHODS

Bacterial strains The Gram negative strains studied were isolated at the Clinical Bacteriological Labora

tory of Umeå, Sweden All strains were from urine cultures ($>10^5$ cells/ml) The urine specimens were kept at about + 4°C during transport They were classified according to standard methods (24) as *Escherichia coli* (E c), *Klebsiella/Enterobacter*, *Proteus* (Pr), *Pseudomonas*, etc

In the conjugation experiments two mutants of the F^- *E coli* K-12 strain RG711 (*proA*, *trp*, *his*, originating from Dr A Datta) were used as recipients 1 RC712, a spontaneous highly streptomycin resistant mutant 2 RC713, an NNG (N nitroso N methyl N-nitroguanidine) induced mutant, resistant to 250 µg/ml of nalidixic acid Mutagen treatment of RC711 bacteria was performed for 30 min at 37° C using fresh log phase bacteria (5×10^8/ml) suspended in citrate buffer (0 05 M, pH 5 5) containing 400 µg/ml of NNG

Growth media The complete medium used was LB of *Bertani* (4) It was solidified with 1 5 per cent agar to obtain LA plates Medium E (25) was the minimal medium used It was supplemented with 0 2 per cent glucose, 1 µg/ml of thiamine and 25 µg/ml of the L-epimer of the amino acids required Minimal plates were obtained by adding 1 5 per cent agar

Chemicals NNG was from K & K Laboratories, Inc, Hollywood, Calif, U S A, D ampicillin and sulphonamide from AB Astra Södertälje, Sweden, streptomycin (sulphate) and chloramphenicol from AB Kabi Stockholm, Sweden nalidixic acid from AB Winthrop, Stockholm, Sweden The tetracycline used was Terramycin® (oxytetracycline), AB Pfizer, Nasbypark, Sweden

Determinations of antibiotic resistance For screening purposes a paper disc method was used (8) For more accurate determinations of resistance levels the bacteria were grown overnight in nylon microculture containers After dilution in 0 9 per cent NaCl using a replicator with steel needles one drop from each culture (about 50 cells) was replicated onto LA plates containing different concentrations of the antibiotic (minimal plates were used for sulphonamide) This equipment was communicated by Dr *G Bertani* and its use in antibiotic resistance determinations was recently described in detail (5) For all antibiotics the following levels were used in the replication resistance test 5, 10 25, 50, 100, 250, 500 and 1000 µg/ml The resistance level was defined as the maximal antibiotic concentration permitting 100 per cent survival of the replicated cells Strains tolerating >25 µg/ml of an antibiotic in this test was considered resistant

Assay of R factor transfer The resistant donor strain was grown in a tube with LB at 37°C These growth conditions optimize sex pilus formation and conjugational chromosome transfer (6) The recipient bacteria were grown in LB under vigorous aeration In the exponential growth phase (4×10^8 cells/ml) 0 05 ml of donor cells and 0 5 ml of recipient cells were mixed in a tube and allowed to conjugate for 2 hrs at 37°C 5 ml of LB was added and conjugation and phenotypic expression were allowed to continue during overnight incubation at 37°C The overnight mating mixture was diluted 10^2 and 10^4 times 0 1 ml portions of undiluted and diluted mating mixture were spread on the selective plates which were incubated overnight at 37°C Transfer of resistance was tested separately for each antibiotic The selective LA plates contained in addition to the antibiotic used for counter selection, 25 µg/ml of D ampicillin (Amp), streptomycin (Str), tetracycline (Tc) chloramphenicol (Cml) or nalidixic acid (Nal) Sulphonamide (Sul) resistant recipient bacteria were always selected on minimal plates usually containing 50 µg/ml of sulphonamide and the antibiotic used for counterselection In most cases strain RC713 was used as recipient using 25 µg/ml of nalidixic acid to counterselect the donor strain When the donor strain was resistant to nalidixic acid it was counterselected by 25 µg/ml of streptomycin using RC712 as recipient strain In a few cases of moderate donor resistance to both streptomycin and nalidixic acid the resistant strain was crossed to both RC712 and RC713 Here higher concentrations of streptomycin (1000 µg/ml) and nalidixic acid (250 µg/ml), respectively were used for efficient counterselection

RESULTS

Antibiotic resistance of the Gram negative bacteria The bacteria used in this study were isolated from routine urine specimens obtained at the Clinical Bacteriological Laboratory of Umeå During a period of the winter 1970/1971 every tenth urine specimen that contained $>10^5$ Gram negative bacteria per ml (11, 12) was analysed by the paper disc method for resistance to the following antibiotics ampicillin, chloramphenicol nalidixic acid, streptomycin, sulphonamides and tetracycline We did not select the bacteria by any clinical criterium Hence, we cannot correlate the bacteria under study to verified urinary tract infection of patients Fig 1 shows that 32 per cent of the strains were sensitive, 27 per cent were resistant to one, 14 per cent to two while 27 per cent were resistant to three or more of the antibiotics under study

The antibiotic resistance of a number of

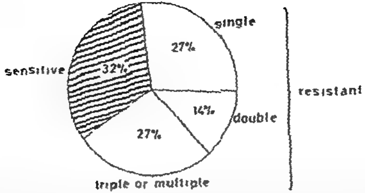

Fig 1 Frequency of sensitivity single double and triple or multiple antibiotic resistance among Gram negative bacterial strains isolated from urine specimens 400 strains were subjected to a disc resistance test (reference 8) Strains giving zone diameters corresponding to the following (or higher) MIC s were regarded as resistant Amp 4 Cml 16 Nal 25 Str 16 Sul 10 and Tc 4 µg/ml (Abbreviations see Materials and methods)

strains was also determined using the more accurate plate dilution method (see Methods) On the basis of such tests we selected 100 single 86 double and 100 multiple (triple or more) resistant strains for further investigation *E coli* was the predominant species (about 50 per cent of the total material) *Proteus* comprised about 40 per cent while the rest represented essentially *Klebsiella/Enterobacter* but a few strains of *Serratia* and *Pseudomonas* were also found The latter genera were rather rare and the discussion will be concentrated on *E coli* and *Proteus* or in most cases on the total collection of bacteria since the objective of this investigation was to estimate the occurrence of R factors

Frequence of non transferable and transferable drug resistance The 286 strains together comprised about 700 resistance traits The distribution of the latter is shown in Fig 2 Tc r represented about 30 per cent Amp r Str r Sul r about 20 per cent each Cml r 7 per cent and Nal r 5 per cent Fig 2 also illustrates to what extent the different resistance traits were transferable under the experimental conditions used About every third instance of resistance to Tc Amp Str or Sul was due to R factors The proportion of transferable Cml r was slightly lower while Nal r was never found to be transferable Of the total material 75 strains out of 286 (26 per cent) carried transferable resistance

The three *Pseudomonas* strains although multiple resistant, did not transfer any antibiotic resistance traits to *E coli* K 12 in our experiments

Levels of non transferable and transferable antibiotic resistance We also determined the approximate levels of antibiotic resistance for all strains Fig 3 shows the distribution of re

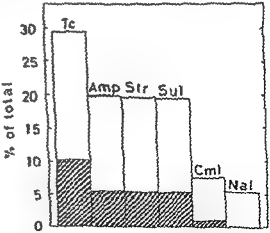

Fig 2 Frequency of occurrence of resistance traits 286 drug resistant strains comprising about 700 resistance traits were studied Hatched parts—transferable traits Abbreviations see Materials and Methods

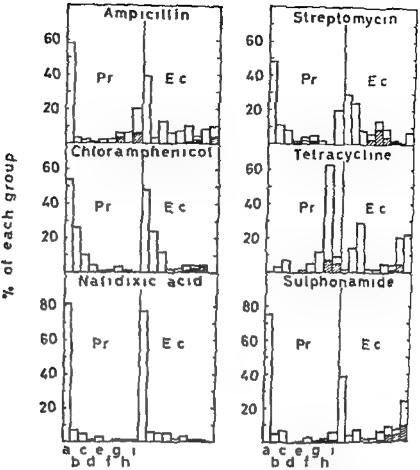

Fig 3 Distribution of levels of antibiotic resistance within each species and for each antibiotic The figures refer to percentages of the total number of strains of each species Hatched parts—transferable resistance a 0 b 5, c 10, d 25 e 50, f 100, g 250 h 500 and i 1000 μg/ml of the respective antibiotic Abbreviations Pr *Proteus* and E c, *E coli* Resistance was determined using the plate dilution method (see Materials and Methods)

sistance levels for the *E coli* and *Proteus* strains and for each antibiotic

A majority of the strains in Fig 3 were sensitive to 5 μg/ml of Nal A similar pattern, although with an increased proportion of resistant strains was seen with Cml Strains resistant to higher levels of Amp, Str, Tc and Sul were commonly found among *E coli* while a majority of the *Proteus* strains were sensitive to Amp, Sul and Str However, *Proteus* organisms were invariably resistant to Tc

Fig 3 also illustrates the proportion of transferable resistance at each resistance level Besides the absence of transferable Nal r mentioned above, it is striking that the R-factors usually mediated resistance to very high concentrations of the antibiotics tested An exception was the level of R factor mediated Str r which was always somewhat lower (50–250 μg/ml) In contrast to the non transferable drug resistance patterns the R-factor mediated resistance patterns showed no significant species differences (*E coli* versus *Proteus*)

Efficiency of R factor transfer The apparent efficiency of R-factor transfer was roughly estimated in each mating The usual frequency of R factor containing recipient cells was 10^{-2}–10^{-3} in the overnight mating mixture The transfer from *Proteus* was often below this range No correlation between

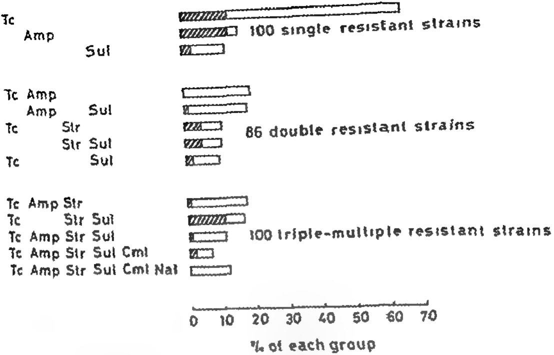

Fig 4 The most common resistance traits or combinations of traits within the single double and triple—multiple resistance groups Hatched parts—transferable traits

transfer frequencies and the resistance pat terns transferred was found

Patterns of non transferable and transferable drug resistance The most common drug resistance traits or combinations of resistance traits are shown in Fig 4 The figure also il lustrates the proportion of transferable drug resistance in each case Although Tc r was dominating in the single resistance group, Amp r and Tc r were transferred equally often In the double resistance group no re sistance combination was predominant Tc r/Str r and Str r/Sul r were the combinations most often transferred whereas Tc r and Amp r were seldom found to be transferred together The multiple group showed a variety of drug resistance combinations of which the five most common are included in Fig 4 Tc r/Str r/Sul r was the dominating R factor combination.

The most common episomal resistance traits or combinations of traits found in these investigations are ranged in Fig 5 It is apparent that double and multiple episomal drug resistance is almost as common as single episomal resistance It should be observed that the transferable resistance combinations indicated in some cases may be due to the presence of more than one R factor in a donor strain In most instances however, the transfer efficiency was similar for all

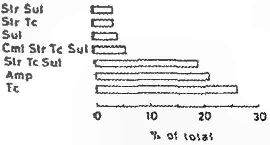

Fig 5 The most common transferable resistance traits or combinations of traits among Gram nega tive bacterial strains isolated from urine specimens The frequency of transferable drug resistance with in the single double and triple—multiple resistance groups (see Fig 4) have here been corrected ac cording to the size of each group (see Fig. 1)

34*

resistance traits, indicating that they might be linked to one genetic unit

Table 1 Drug Resistance and Transferable Drug Resistance among Strains from Patients of Different Age and Sex

Patient* group (years)	Frequency of resistance**			
	Total°		Transferable §	
	No	%	No	%
> 50	106	78	43	27
11–50	28	53	9	32
1–10	5	22	6	40
< 1	9	48	3	15
Male	61	75	28	26
Female	89	65	33	31

* *The clinical diagnosis of the patients was not traced*

** Resistance to at least one of the antibiotics studied

° Based on 250 urine cultures subjected to disc resistance tests

§ Based on findings in 220 patients whose drug resistant urinary bacteria were studied further in this investigation

Sources of bacteria with transferable and non transferable antibiotic resistance The sources of 249 of the 286 resistant strains investigated could be traced These 249 strains originated from urine specimens of 220 different patients As seen in Table 1 the frequency of antibiotic resistant strains was lower in age group 1–10 years (22 per cent) compared to 50–70 per cent in higher age groups R-factor carrying strains were found in all age groups Roughly every third antibiotic resistant strain was capable of transferring resistance to *E coli* K 12 No drastic differences between men and women were found in any of these respects

Table 2 shows the frequency of drug resistant strains and of R factor carrying strains among specimens from different wards as well as among specimens from outpatients In most cases (including outpatients) 60–75 per cent of the strains were antibiotic resistant The paediatric clinic represented an exception, about 30 per cent resistant strains being demonstrated there The frequency of episomal resistance among the antibiotic resistant strains was similar in all clinics (26–36 per cent) Among specimens from outpatients this figure was somewhat lower (21 per cent)

Table 2 Drug Resistance and Transferable Drug Resistance among Strains from Patients in some Larger Clinics

Patient* from	Frequency of resistance**			
	Total°		Transferable §	
	No	%	No	%
Internal medicine	35	71	18	36
Chronical ward	29	76	13	27
Urology	11	72	7	26
Surgery and gynaecology	18	60	7	26
Paediatrics	22	32	9	39
Outpatients	34	72	6	21

* The clinical diagnosis of the patients was not traced

** Resistance to at least one of the antibiotics studied

° *Based on 250 urine cultures subjected to disc resistance tests*

§ Based on findings in 220 patients whose drug resistant urinary bacteria were studied further in this investigation

DISCUSSION

First it should be emphasized that the bacteria studied were not selected by any clinical criterium Routine urine specimens for bacteriological test were *investigated* Although the specimens tested contained $> 10^5$ bacteria/ml a portion of the bacteria isolated may have been contaminants The objectives of this investigation was however, to study the occurrence of R factors and antibiotic resistances among Gram negative bacteria isolated from urine specimens *The investigation* shows that antibiotic resistant bacteria are common in urinary specimens in Northern Sweden as in many other places throughout the world 68 per cent of the urine cultures contained drug resistant strains many of which were resistant to several of the anti

biotics studied here. Resistance was most common for Tc, less common for Amp, Str and Sul and least common for Cml and Nal.

In 26 per cent of the drug resistant strains resistance was R-factor mediated. This figure was somewhat higher for the *E. coli* strains and lower for the *Proteus* organisms. The frequency of R factors found is lower than that observed in a similar investigation in Norway (45 per cent, reference 1) and significantly lower than in a survey in Boston, Mass., U.S.A. (69 per cent, reference 18).

The incidence of R factors found represents a minimal estimate for the following reasons: a) lack of drug resistant progeny in our mating experiments may be due to production of bacteriocin by the donor cells thus killing the recipients (17). Bacteriocin production is common among wild Gram negative strains (reference 17, *Bergfors & Eklöf*, unpublished observations). b) Some R-factors may have lost their sex part (RTF, resistance transfer factor, reference 3), thereby being unable to transfer themselves to other bacterial cells. c) Inefficient transfer may be due to incompatibility between the donor and the recipient cells. The basis for varying compatibility is not known but may involve cell surface differences impairing mating pair formation. Low transfer efficiency was frequently obtained from *Proteus* to *E. coli* K-12. So far, there is only one case known of transfer of R-factor from *Pseudomonas* to *E. coli* (19). d) Inability of certain R factors to replicate in *E. coli* K 12 cells. It is thought that episomes (like R factors) as well as the bacterial chromosome replicate attached to distinct sites on the cytoplasmic membrane (10). Consequently competition for the same replication site may explain why certain R-factors cannot co-exist in one host bacterium (23). In this investigation, Amp-r and Tc-r were often found to be transferred separately, but joint transfer of Amp-r and Tc-r was only rarely observed. This is in contrast to many other studies and may be due to the particular *E. coli* K-12 strain used. However, *Anderson* (2) has reported several cases in which different resistance traits are present on separate DNA-molecules. e) Foreign DNA is often restricted in bacteria.

Although there were certain differences between *E. coli* and *Proteus* in overall resistance patterns, the R-factor mediated resistance patterns were similar in both species. One exception was that *Proteus* transferred few resistance traits but Amp-r and Tc-r. The species similarities of R-factor patterns indicate that the same or similar R factors are spread in a variety of host bacteria. As in earlier investigations, the R factors studied here mediated very high resistance levels to all antibiotics except for Str where the levels were moderately high (50–250 μg/ml).

The origin of R factors is still not known. It has been proposed that single RTF-factors could "pick up" resistance genes from the bacterial chromosome (22) and such factors have been found in wild bacteria (2). However, there is little direct evidence in favour of this hypothesis. Analyses of R-factor DNA have shown a great heterogeneity in base composition. This indicates that R-factors have multiple origins being products of repeated genetic recombination between factors carried by the same cell (9).

No doubt, the use of antibiotics is an important selective pressure increasing the incidence of drug resistant microorganisms. The frequency of drug resistant strains were in this study equal among urine specimens from inpatients and outpatients. R-factor strains were somewhat more common among drug resistant strains from Umeå hospital (29 per cent) than strains from urine of the outpatients (21 per cent). Strains from the same ward could be seen to carry the same transferable resistance pattern suggesting that cross-infection may be common. On the other hand, the relatively high frequency of transferable and non transferable drug resistance among the specimens from outpatients and the fact that resistance to antibiotics not used as first choice in man often was formed (Str, Cml) could indicate that many R factors have been selected and spread not only in hospital environments but also through the use of antibiotics in animal

breeding (15, 16, 21) Tetracyclines are the only antibiotics tested in this study that are allowed as additives in animal breeding in Sweden

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GROWTH INHIBITION AND CELL ENLARGEMENT IN P-388 CELLS TREATED WITH MITOMYCIN C

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The effects of mitomycin C on growth and cell size in shake flask cultures of P-388 mouse cells have been investigated. Concentrations of the antibiotic in the range 0.01 to 1.0 μg/ml progressively inhibited cell growth. With 0.5 μg/ml of mitomycin C, cell multiplication stopped after 1 day and an increasing fraction of the cells were dying, reaching 20 per cent after 3 days' treatment. At least 50 per cent of the cell population grew into giant cells. Exposure for 1 hour to 0.5 μg/ml of the antibiotic caused a slight inhibition of cell multiplication and a significant increase in giant cell formation after 1 day. Upon further incubation these effects were overcome. Increased mean cell size was also found 1 day after this treatment, followed by a return to a normal cell size distribution.

Mitomycin C has been shown to inhibit growth of a wide variety of microorganisms and mammalian cells, and the effect has been ascribed to an alkylation of DNA (*Szybalski & Iyer* 1967).

In bacteria exposed to the antibiotic indications for DNA repair has been found, similar to the repair of DNA damage induced by irradiation and alkylating agents (*Strauss* 1968, *Kersten & Kersten* 1969). Recent data (*Rauth et al* 1970) might indicate that repair of mitomycin C induced damage occur also in mammalian cells.

As mouse lymphoma P-388 cells have been found suited for a study of DNA repair synthesis after irradiation (*Ayad & Fox* 1969, *Fox et al* 1970), these cells might prove useful in studies on DNA repair mechanisms in mitomycin C treated mammalian cells. As a first step the effects of the antibiotic on growth and size of the P-388 cells grown in culture have been studied.

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MATERIALS AND METHODS

P 388 cells, originally derived from a mouse lymphoid neoplasm (*Dunn & Potter* 1957), were maintained as shake flask cultures on Eagle's minimum essential medium (*Eagle* 1959) with 10 per cent foetal bovine serum. The viscosity of the medium was raised by adding 1.2 g methocel per litre to prevent cell destruction (*Earle et al* 1954), and pluronic acid (1 g/l) was added to prevent fibre formation (*Swim & Parker* 1960). Penicillin (100 I.U./ml) and streptomycin (100 μg/ml) were used.

Cell multiplication was followed by counting in a haemocytometer and in an electronic cell counter (Celloscope 202). The electronic counter was calibrated against pollen of a known diameter (12.6 μ), and counts due to particles smaller than 800 μm^3 were excluded to avoid interference from subcellular particles and electronic noise.

Normal cell size distribution was determined by counting particles of increasing volumes in steps of

200 μm^3 in the range 800 3000 μm^3 · *Giant cell formation* was followed by counting particles larger than 3000 μm^3 and further increase in cell size de termined by counting particles exceeding 4000 μm^3

Cell death was estimated by staining with try pan blue (*Tennant 1964*) and by measuring the *release of radioactivity* to the culture medium from cells prelabelled with ^3H thymidine Radioactivity was measured in a Packard Tri Carb model 3365 liquid scintillation counter

Chemicals

Mitomycin C (Kyowa Hakko Kogyo Co Ltd, Tokyo) was kept as aqueous stock solution (1 mg/ml) at 4° C and used within one week Thymidine 6 T(n) (3 5 Ci/m mol the Radiochemical Centre Amersham) was kept in aqueous stock solution (*100 μCi/ml*) at 4° C and used within two months · Scintillation liquid consisted of 2 5 di phenyloxazole 4 g 1 4 bis 2 (4 methyl 5 phenyl oxazolyl) benzene 0 05 g toluene ad 850 ml etyleneglycol monomethyl ether ad 1000 ml

RESULTS

Effects of Continuous Exposure to Mitomycin C

Cell growth The effect of mitomycin C on growth of P 388 mouse cells was measured by growing the cells in medium containing vary ing concentrations of the antibiotic The re sults from these experiments are shown in Fig 1 With a given drug concentration the degree of inhibition varied to some degree but within a single experiment the cell multiplication was progressively suppressed with increasing concentrations of the anti biotic up to 1 0 μg/ml culture (Fig 1a) With all concentrations tested the cell num ber increased during the first day of treat ment After this time the number of cells in cultures exposed to 1 0 μg/ml of mitomycin

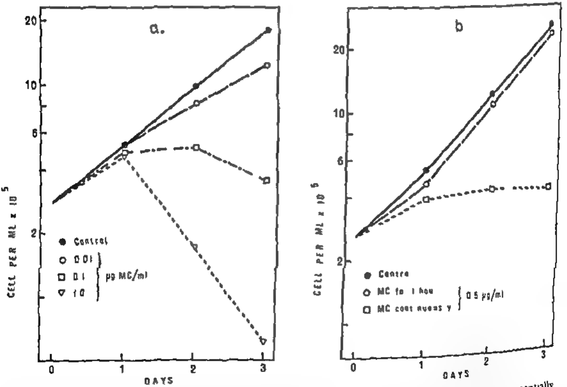

Fig 1 Effect of mitomycin C on cell growth Suspension cultures were prepared from exponentially growing cells and diluted daily with fresh medium Cultures treated continuously were diluted with medium containing mitomycin C (MC) in the concentrations indicated Cells treated for *1 hour* were harvested by centrifugation washed and grown further in medium without MC Mean cell counts from two parallel cultures have been recorded

a Haemocytometer counts from continuously treated cultures

b Electronic cell counts after continuous and 1 hour treatment with 0 5 μg/ml of MC

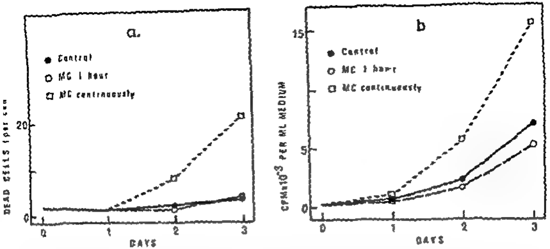

Fig 2 Effect of mitomycin C on cell vitality Suspension cultures were prepared from cells prelabelled with ^3H thymidine After incubation for 1 hour with and without 0 5 μg/ml of mitomycin C (MC) the cells were harvested by centrifugation and washed Controls and one half of the cells exposed to MC were then incubated in medium without MC the rest in medium containing 0 5 μg MC/ml The cultures were diluted daily with the respective media The mean values from two parallel cultures have been recorded

a Per cent of the cells heavily stained with trypan blue

b Transfer of radioactive material from the cells to the culture medium

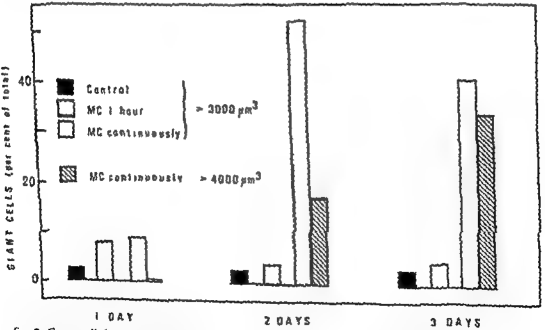

Fig 3 Giant cell formation in mitomycin C treated P 388 cell cultures Exponentially growing cells were incubated with and without 0 5 μg/ml of mitomycin C (MC) for 1 hour harvested by centrifugation and washed Controls and one half of the cells exposed to MC were then incubated in medium without MC the rest in medium containing 0 5 μg MC/ml The cultures were diluted daily with the respective media Cells exceeding 3000 μm^3 and 4000 μm^3 were counted with an electronic cell counter, and the results have been recorded as per cent of the total number of cells in the culture

C decreased rapidly, with intermediate concentrations (0 1 and 0 5 μg/ml) the cell count remained fairly stable, and in cultures treated with 0 01 μg/ml the growth rate was only slightly reduced compared with the controls

Cell death Cell death was measured after 1, 2 and 3 days of cultivation in the presence of 0 5 μg/ml of mitomycin C The results in Fig 2a show that this treatment did not induce any change in the number of non-vital cells during the first day After that time an increasing fraction of the cells were dying, reaching 20 per cent of the population after 3 days of treatment

Non-vital cells may exclude trypan blue and thus be recorded as living cells with this method (*Tennant* 1964) The data in Fig 2a, however, were supported by measurements of the release of radioactivity from mitomycin C treated cells prelabelled with ^{3}H-thymidine (Fig 2b)

Giant cell formation The effect of mitomycin C on the formation of giant cells was measured in cultures treated with 0 5 μg/ml of the antibiotic Exponentially growing control cultures contained a limited number of giant cells as defined here Thus only 2–3 per cent of a normal cell population had a volume exceeding 3000 μm^3 It can be seen from Fig 3 that the fraction of cells larger than 3000 μm^3 increased to more than 50 per cent of the total cell count after 2 days' treatment with mitomycin C, and then fell to about 40 per cent after 3 days It appears from the graph, however, that the per cent of the total cell number in the cultures exceeding 4000 μm^3 continued to increase also during the third day of treatment

Effects of Temporary Exposure to Mitomycin C

The effects of temporary exposure of P 388 cells to mitomycin C were measured by incubating the cells with 0 5 μg/ml of the antibiotic for 1 hour, followed by further incubation in medium without mitomycin C It appears from Fig 1b that cell multiplication was only slightly suppressed by this treatment, and no signs of cell death were found (Fig 2) The fraction of giant cells in the cultures increased to 8 per cent 1 day after the treatment (Fig 3), but upon further incubation of the cultures the number of giant cells returned to nearly normal values

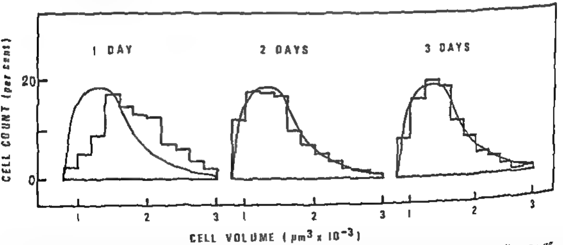

Fig 4 Cell size distribution of P 388 cells treated with mitomycin C for 1 hour Exponentially growing cells were incubated in medium with and without 0 5 μg/ml of mitomycin C (MC) for 1 hour harvested by centrifugation washed and further incubated in medium without MC Cells with volumes ranging from 800 to 3000 μm^3 were counted with an electronic cell counter Cell size distribution patterns from control cultures (curved lines) and from cultures exposed to MC for 1 hour (histograms) were recorded after 1 2 and 3 days

Fig 4 shows a more complete cell size distribution pattern from control cultures and from cultures which had been treated with mitomycin C for 1 hour (0 5 μg/ml) The mean volume of the control cells was 1450–1500 μm^3 whereas the treated cells had a mean volume above 1800 μm^3 1 day after the treatment After 2 and 3 days the cell size distribution in the treated cultures was the same as in controls

DISCUSSION

Growth inhibitory concentrations of mitomycin C for P 388 cells in suspension culture of 0 1–0 5 μg/ml corresponds well with previous results obtained for other mammalian cells grown as monolayer cultures (*e g Shatkin et al* 1962 *Magee & Miller* 1962 *Cohen & Studzinski* 1967) Growth curves as determined by cell counts (Fig 1) might reflect a balance between cell death and cell division in the cultures It appears that the growth inhibition of the P 388 cells treated with 0 5 μg/ml of mitomycin C (Fig 1b) was primarily due to reduced cell division because the number of dying cells was low initially (Fig 2) As the cell count remained stable also after significant cell death occurred however a limited fraction of the cells apparently continued to divide This is in agreement with a previous study on mouse L cells (*Rauth et al* 1970) A small fraction of these cells formed colonies in the presence of 0 3–0 5 μg/ml of the antibiotic

Cultivation for several days implied variations in the acidity of the culture medium and the pH of the medium has been shown to influence the cytotoxicity of mitomycin C for other mammalian cells (*Rauth et al* 1970) This factor might explain varying degrees of inhibition caused by a given drug concentration and also the overlap of growth inhibition caused by 0 1 μg/ml of the antibiotic in one experiment (Fig 1a) and by 0 5 μg/ml in another (Fig 1b)

Substantial increase in the cell number during the first day of treatment (Fig 1) can be explained by assuming that mitomycin C attacked the cells at a certain stage in their cell cycle allowing cells past this stage to proceed through the following cell division before they were arrested This view is supported by observations on other mammalian cells Thus HeLa cells treated with the antibiotic for 1 hour showed unchanged mitotic index for a period of 6 hours indicating that cells exposed to mitomycin C in the last stages of their cell cycle entered the following mitosis (*Doi et al* 1967) Also, human leucocytes showed nearly unchanged mitotic activity when they were exposed to mitomycin C for 2–4 hours in G phase (*Nowell* 1964) Since *in vitro* studies have shown that mitomycin C must be activated prior to alkylation of DNA (review *Szybalski & Iyer* 1967) a delayed activation in P 388 cell cultures cannot be ruled out as a factor contributing to the lag period

Extensive giant cell formation of the P 388 cells grown in suspension with 0 5 μg/ml of mitomycin C is in agreement with previous observations on other cells grown as monolayer cultures (*Shatkin et al* 1962 *Cohen & Studzinski* 1967) Giant cell growth in the presence of mitomycin C has been explained as unbalanced growth with continued synthesis of RNA and protein after inhibition of DNA synthesis and blocked cell division (*Szybalski & Iyer* 1967) After more than 2 days of treatment the fraction of giant P 388 cells (exceeding 3000 μm^3) decreased (Fig 3) probably as a result of cell death (Fig 2) On the other hand unbalanced growth continued apparently as the fraction of cells exceeding 4000 μm^3 increased also during the third day (Fig 3)

The data from cultures exposed to mitomycin C for 1 hour (0 5 μg/ml) show that this treatment reversibly altered the growth of the P 388 cells The slight reversible suppression of the growth rate (Fig 1b) was not caused by selective killing of a minor fraction of the cells (Fig 2), or by irreversible transformation of a few cells into giant cells (Fig 3) As the average cell size in these cultures temporarily increased 1 day after the treatment (Fig 4) the most likely ex

planation seems to be that the exposure to mitomycin C had induced unbalanced growth and that the P-388 cells possessed a system for repair or circumvention of the initial drug effects

Considering the central role of DNA as a target for mitomycin C (*Szybalski* & *Iyer* 1967) and the recent suggestion that damage caused by the antibiotic in mouse cells may be repaired (*Rauth et al* 1970), the present results might suggest that mechanisms exist in the P-388 cells for repair of mitomycin C induced damage of DNA Further work on the effect of mitomycin C on DNA metabolism in P-388 cells is in progress

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A COMPARISON OF PHAGE PATTERN AND ANTIGENIC STRUCTURE WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM SWINE

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Seventy-one strains of *Staphylococcus aureus* isolated from the nasal cavities of healthy swine were studied biochemically, serologically and by phage typing. Sixty-nine strains (97 per cent) were typable by phages of the International Basic Set: 55 strains at RTD and 14 strains at RTD × 1 000, whereas only 30 strains were lysed by bovine phages from the provisional set (Davidson's set). Sixty-six strains (93 per cent) were typable with factor sera. The agglutinations were strong and usually only one type agglutinogen was present. Fifty strains formed a homogeneous group with biological properties considered as characteristic of staphylococci occurring in swine. These strains were of crystal violet type A, fibrinolysin negative, produced β haemolysin and pigment, coagulated human plasma strongly but not bovine plasma. They belonged to phage group II and had the c_1 agglutinogen.

The environment of the host is probably the selective factor which determines the whole complex of biological properties in the adapted population of staphylococci. The separation of ecologically different strains of *Staph. aureus* is first of all based on their biochemical examination. The determination of the sensitivity to phages and the antigenic structure contributes however considerably to a more detailed characterization of strains and enables a more exact classification of staphylococci of different origin.

In the present report biochemical properties, sensitivity to phages and antigenic structure have been correlated in staphylococci isolated from swine.

MATERIAL AND METHODS

Seventy-one coagulase-positive staphylococcal strains were obtained by means of swabs from the nares of 90 swine from different areas of Northern Moravia, Czechoslovakia. The specimens were collected immediately after the animals had been killed in the slaughter house of Olomouc.

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The strains were tested for the following biochemical characteristics Mannitol fermentation coagulation of human bovine and porcine plasma the clumping factor, the production of pigment haemolysins fibrinolysin, the egg yolk factor and some other enzymes the growth type on crystal violet agar, and the sensitivity to antibiotics and sublimate The results of the biochemical examinations have been presented in detail in (5)

Phage typing was carried out according to the method of *Blair & Williams* (1961) All strains were tested with the phages of the International Basic Set for the typing of human staphylococci (18), and with the provisional phage set proposed by *Davidson* (1966), the latter set being composed of phages 29 52A 3A, 6, 53, 75 77, 84 42D from the International Basic Set and 'bovine' phages AC_1 116 102, 107, 117, 118, 119, 78 Strains not typable with the routine test dilution (RTD) were retested with RTD × 1 000

Slide agglutination was performed according to the method of *Oeding* (1957) with the following factor sera a_1 a, b_1 c_1, e, h_1 h, $i_1 i_2$ k_1 $k_1 k$ m, n *263–1*, and *263–2* All antisera were prepared by immunization of rabbits with human *Staph aureus* strains For technical details see also (7) The precipitinogens were examined by the double diffusion technique in agar gel (8) The strains were tested with immune sera prepared against the human *Staph aureus* strains Wood 46 and Cowan I Polysaccharide A and protein A were included as references (9 16)

RESULTS

Biochemical examinations

Of the total number of 71 staphylococci isolated from the nares of swine 37 strains (52 per cent) produced yellow pigment, 26 strains (37 per cent) orange pigment and 7 strains (10 per cent) cream coloured pigment

Yellow pigmented staphylococci behaved as a homogeneous group according to their biochemical properties All except one were of crystal violet type A (yellow) they were resistant to tetracycline and sensitive to penicillin (Table 1)

The group of 33 orange and cream pigmented staphylococci had relatively heterogeneous characteristics Only 14 of these strains were of crystal violet type A whereas 19 belonged to crystal violet type C (violet) Tetracycline resistance was shown in 27 strains 10 strains being also resistant to penicillin The penicillin resistant isolates belonged predominantly to crystal violet type C (Table 2, Table 3)

One strain did not produce any pigment and differed considerably from the whole set of porcine isolates by its small biochemical activity This strain was therefore designated as atypical

The sublimate resistance of the strains (47 per cent) is assumed to be linked to the resistance to tetracycline (90 per cent)

Altogether, the biochemical activity of these staphylococci of porcine origin was relatively high (5) Their main characteristics were the absence of fibrinolytic activity, the production of pigment and β haemolysin the coagulation of human but not bovine plasma and that they were of the crystal violet type A

Phage Typing

Sixty nine (97 per cent) of the strains were successfully typed by the phages used 55 strains at RTD and 14 strains at RTD ×1,000 (Fig 1)

The yellow pigmented strains also formed a homogeneous group with regard to phage patterns All these strains were typable by phages of the International Basic Set at RTD An absolute majority (84 per cent) belonged to group II the main phage pattern being 3A/3C/55 or 3A/55 Only 4 strains belonged to the mixed group II/III 1 strain belonged to group I and 1 strain (*V 17/68 OL*) behaved atypically being lysed by almost all phages except by those of group *II* This strain was the only one of the yellow pigmented isolates which was of crystal violet type C With Davidson's phage set 35 strains were sensitive to phage *3A* 20 of these also to the 'bovine' phage *116* which like phage 3A belongs to phage group II (Table 1) One strain remained untypable

Also in the orange and cream pigmented staphylococci the sensitivity to phages was in close correlation with the crystal violet type The sensitivity to phages of the strains of crystal violet type A was similar to that of

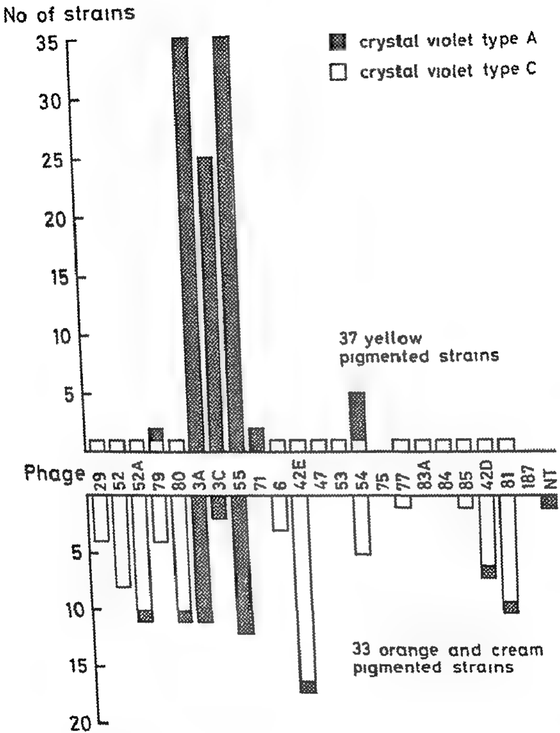

Fig 1 Sensitivity of 70 *Staph aureus* strains to phages of the International Basic Set correlated to pigmentation and crystal violet type The results of phage typing include only strong lytic reactions (> 50 plaques—confluent lysis)

TABLE 1 *Phage and Antigen c Pa*

Crystal violet	Phage pattern: International Basic Set	Provisional set: Human phages	Provisional set: Bovine phag
Type A (yellow)	3A/3C/55/54	3A	118/116
	3A/3C/55/54	3A	116
	3A/3C/55/71	3A	116
	3A/3C/55	3A	116
	3A/3C/55	3A	116
	3A/3C/55	3A	116
	3A/3C/55	3A	—
	3A/55	3A	116
	3A/55	3A	—
	3A/55	3A	—
	79	NT	NT
Type C (violet)	29/52/52A/79/80/6/42E/47/53/54/77/83A/84/85/42D/81	29/52A/6/53/77/84/42D	118/AC$_1$/117/10 107

The results of phage typing include only strong lytic reactions (50 plaques—confluent lysis)
PC = penicillin TC = tetracycline

TABLE 2 *Phage and Antigenic Patt*

Crystal violet	Phage pattern: International Basic Set	Provisional set: Human phages	Provisional set: Bovine" phages
Type A (orange)	52A/80/42E/42D/81	52A/42D	—
	3A/3C/55	3A	—
	3A/3C/55	3A	—
	3A/55	3A	116
	3A/55	3A	116
	3A/55	3A	116
	3A/55	3A	—
	3A/55	3A	—
	3A/55	3A	—
	55	NT	NT
	NT	NT	NT
Type C (violet)	29/52/52A/80/6/85/42D/81	29/52A/6/42D	—
	52/52A/80/42E/42D/81	52A/42D	—
	52A/80/42E	52A	—
	52A/80/42E	52A	—
	42E/54/77	77	—
	42E/54/81	—	—
	42E/42D/81	42D	—
	42E/54	NT	NT
	42E	NT	NT

The results of phage typing include only strong lytic reactions (50 plaques—confluent lysis)
PC = penicillin, TC — tetracycline

of Yello c Pigmented Strains

Antigenic pattern	Resistance to PC	Resistance to TC	Number of strains	Total
c_1	—	+	1	
c_1	—	+	3	
c_1	—	+	2	
c_1	—	+	10	
b_1/m	—	+	1	36 strains
NT	—	+	2	
c_1	—	+	6	
c_1	—	+	1	
c_1		+	8	
h	—	+	1	
c_1	—	+	1	
NT	—	—	1	1 strain

of Orange Pigmented Strains

Ai t genic pattern	Resistance to PC	Resistance to TC	Number of strains	Total
c_1	—	+	1	
c_1	—	—	1	
NT	—	+	1	
a /b_1/k_1k /m		+	1	
c_1	—	+	3	
c_1	+	+	1	14 strains
c_1		+	2	
c_1	+	+	1	
h.	—	—	1	
c_1	—	—	1	
c_1	—	+	1	
NT	—	+	1	
h	—	+	1	
h	+	+	3	
h	—	+	1	
h	+	+	1	12 strains
c_1	+	—	1	
h	—	+	1	
h	+	+	1	
h	+	+	2	

Crystal violet	Phage pattern		
	International Basic Set	Provisional set Human phages	Provisional set Bovine phages
Type C (violet)	29/52/52A/79/80/6/42E/42D/81	29/52A/6/42D	118/AC,
	29/52/52A/79/80	29/52A	118/AC,
	29/52/42E/54/81	29	—
	52/52A/79/80/6/42E/54/42D/81	52A/6/42D	118/119/AC
	52/52A/79/80/42E/42D/81	52A/42D	—
	42E/81	NT	NT
	52	NT	NT

The results of phage typing include only strong lytic reactions (50 plaques—confluent lysis)
PC = penicillin TC = tetracycline

strains producing yellow pigment Out of 14 of these strains 12 were lysed by phages of group II, the majority having phage pattern 3A/55 One strain remained untypable With Davidson s phage set the majority of these staphylococci was lysed by phage 3A, some even by phage 116 Two strains were not sensitive to Davidson's provisional set of phages (Table 2)

The staphylococci of crystal violet type C to which belonged 12 strains with orange pigmentation and 7 with cream pigmentation, showed quite different phage patterns Two strains belonged to group I and 4 strains to group III, whereas the remaining 13 strains were lysed by phages of different groups, not including those of group II All these strains except 5, were sensitive to phages from different groups of Davidson s set, but not in a single case to phages 3A and 116 of group II (Table 2 Table 3)

All 14 strains lysed only at RTD×1 000 produced an orange pigment Four strains of crystal violet type A, were sensitive to phages of group II (3A/55, 3A/3C/55) The remaining 10 strains which were of crystal violet type C reacted with phages of different groups but not with group II

Serological Typing

Sixty six (93 per cent) of the 71 strains were typable with the factor sera used The agglutinations were strong and as a rule only one type agglutinogen was demonstrated

In 32 out of 37 yellow pigmented strains (87 per cent) the c_1 antigen only was demonstrated, 2 strains had other antigens and 3 were untypable The serological examination thus gave added evidence of the strong homogeneity of this group of isolates (Table 1)

In the 33 strains with orange and cream pigment the agglutinogen composition was also in close correlation with the crystal violet type and with the results of phage typing Strains of crystal violet type A corresponded by their serological pattern to the group of staphylococci with yellow pigment Out of 14 strains 11 had the c_1 antigen only 2 strains had other antigens and 1 was untypable Of the 19 orange and cream pigmented staphylococci which were of crystal violet type C 12 strains (63 per cent) had the antigen h alone 1 strain had the pattern h /m and only 4 strains had the antigen c_1 (Table 2 Table 3)

The atypical strain which had low biochemical activity was non pigmented and untypable by the phages used had the type agglutinogen c

The distribution of precipitinogens is given totally for all the 71 porcine isolates Polysaccharide A was demonstrated in 66 per cent of the strains whereas only 13 per cent had protein A

f Cream Pigmented Strains

Antigenic pattern	Resistance to PC	Resistance to TC	Number of strains	Total
a_3/k_1k	—	+	1	
c_1	—	—	1	
h_2/m	—	+	1	
h_2	—	+	1	7 strains
h	—	+	1	
c_1	—	+	1	
c_1	—	—	1	

DISCUSSION

The environment of the upper respiratory tract in swine may be an ecological factor leading to the selection of a staphylococcal population with characteristic biological properties The strains of the present material were characterized by the production of β haemolysin and the absence of fibrinolytic activity i e by qualities currently met with in the staphylococci of many animals (3 4, 10 12 13 14) Additional biochemical characteristics of the strains were coagulation of human but not bovine plasma production of pigment clumping factor egg yolk factor and delta haemolysin but only occasionally of α toxin (5)

The high frequency of tetracycline resistant strains is probably connected with the current addition of chlortetracycline to the fodder of swine in a dose of 30 mg per 1 kg It is open to discussion whether there is a connection between the development of resistance to tetracycline and change of pigment Such a connection would suggest that the yellow pigmented strains are the ones best adapted to the host organism

The pigmentation and especially the crystal violet type enabled the selection of a homogeneous group of strains, the characteristics of which seem to be shared by many staphylococci of porcine origin (6) Practically all of the 50 isolates of crystal violet type A to which the strains with yellow pigment and some of the orange pigmented strains belonged, had the same sensitivity to phages and identical antigenic structure

The number of strains typable by phages was remarkably high It was possible to identify 97 per cent of the strains by means of the International Basic Set for the typing of human staphylococci, whereas 42 per cent were typable by means of only the 'bovine" phages from the provisional set (Davidson's set) Of the 50 strains of crystal violet type A 86 per cent belonged to phage group II The main phage patterns were 3A/3C/55 and 3A/55

Mori et al (1970) have published findings from Japan which are very similar to ours They were able to type 81 per cent of 58 staphylococcal strains isolated from the nares of swine by the phages of the International Basic Set 60 per cent having the phage pattern 3A/55 or 3A A relatively frequent occurrence of strains of crystal violet type A belonging to phage group II was also found in healthy swine in the German Democratic Republic by *Horsech & Fiebig* (1965)

A high proportion (93 per cent) of the examined porcine staphylococci was also typable serologically, using factor sera prepared against human strains of *Staph aureus* All the typable strains agglutinated strongly Among the 50 strains belonging to crystal violet type A the c_1 antigen only was demon-

strated in 86 per cent A poor antigenic structure, often expressed by one single agglutinogen, has repeatedly been reported in staphylococci of animal origin (2, 11, 17). The strong agglutinability, as well as the sensitivity to human phages and the relatively high biochemical activity, indicates that this group of porcine staphylococci is more closely related to staphylococci of human origin than are strains from other animals (6)

The remaining 20 strains of crystal violet type C and 1 strain of type E (white) were clearly different from the strains of type A. They did not react with phages of group II, belonging to phage group I, III or to mixed groups. In 57 per cent of the strains the h_e agglutinogen was demonstrated. Both the c_1 and h_e agglutinogen have frequently been found in staphylococci isolated from different animal species (11, 17)

The staphylococci of crystal violet type C may have been acquired from other animal species, some of them even from man. The present investigation thus confirms the observation that the population of staphylococci isolated from one animal species is usually not homogeneous. Strains with a typical complex of biological properties prevail among isolates of one ecological origin, but staphylococci having the characteristics of other site variants or atypical strains, may also be represented to a lesser extent. A thorough examination and interpretation of biochemical properties, in addition to an examination of phage sensitivity and antigenic structure, is therefore absolutely necessary for the differentiation and characterization of staphylococci of animal origin

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CHARACTERIZATION *OF A STRUCTURAL ANTIGEN* OF RUBELLA VIRUS REACTING BY GEL PRECIPITATION

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Two to three gel precipitating (GP) antigens of rubella virus were extracted from purified rubella virus by treatment with the nonionic detergents Nonidet P40 and Tween 20 The strongest of these GP antigens was further characterized This antigen sedimented through a 5–20 per cent sucrose gradient in an ultracentrifuge at an S value of about 3 5 It banded in a CsCl equilibrium gradient at densities of 1 25 to 1 30 g/ml An identical small sized antigen could be extracted from rubella infected BHK 21 cells This antigen seemed to be part of the rubella virus envelope Some characteristics of the remaining core particles were determined

Gel precipitating antigens of rubella have been described (15 16, 19, 20, 22), and some properties of the antigens have been studied (14, 21) *Schmidt et al* suggested that a gel precipitating antigen of rubella was a part of the rubella virus itself (22) But no direct proof of that relationship has been published The properties of a soluble complement fixing and platelet aggregation rubella anti gen released from virions were described by *Vaheri et al* (30) The relationship of the complement fixing, platelet aggregating and gel-precipitating activities of this small sized antigen were described in a preliminary re port (29)

In this report the properties of a gel pre cipitating structural antigen released from purified rubella virus are presented A soluble antigen from rubella infected cells is com pared with the antigen released from the purified rubella virus and the antigenic iden tity of these is shown

Received 24 iii 72

MATERIALS AND METHODS

Virus The RA 27/3 strain (27) of rubella virus was used at the third to tenth passage in this laboratory

Cells The clonal baby hamster kidney cell lines BHK 21/13S and BHK 21/WI 2 (26) were em ployed in this work The suspension culture method used to maintain the BHK 21/13S cells has been described elsewhere (20) The BHK 21/WI 2 cells were grown and maintained in a roller drum (Assab/Gothenburg Sweden), using bottles of 0 5 litre capacity At the time of virus inoculations ontained one les had a sion cul tures was 400 ml or 800 ml and the lower bottles contained 20 ml of medium each Both types of cul tures were maintained in the BHK medium (Grand Island Biological Co New York) supplemented with 5 per cent inactivated foetal calf serum or 0 4 per cent bovine serum albumin fraction V (Armour Pharmaceutical Co Chicago Illinois)

Buffers and chemicals PBS buffer Dulbecco's phosphate buffered saline without Mg and Ca, pH 7 4

TEN buffer Tris EDTA saline buffer 0 04 M Tris, 0 001 M ethylendiamine tetra acetate (EDTA) 0 15 M NaCl pH 7 4

Nonionic detergent Nonidet P40 (NP40) was a gift from the Shell Chemical Company

Tween and Span detergents were purchased from the Atlas Chemical Industries S A, Belgium

Sodium dodecyl sulphate (SDS) was recrystallized according to *Mandel* (17) Stock solutions of desoxycholate (DOC) and sodium periodate dissolved in PBS were made up without any purification

Purification of rubella virus Cell cultures were infected at a multiplicity of infection of 1, adsorbed at +37° C for 90 minutes and incubated in the maintenance medium at +35° C for 48 to 60 hours (BHK 21/13S cells) or 40 to 48 hours (BHK 21/WI 2 cells) Supernatants from the cultures were harvested by spinning down the cells at 1000 g for 15 minutes The cells were used as starting material for cell-derived soluble antigen and the supernatant was immediately processed to obtain the purified virus

The supernatant from BHK 21/13S cells was first concentrated by vacuum dialysis at +4° C, using Visking dialysis tubings and plastic water jet pumps (Edwards High Vacuum Ltd, Sussex England) The concentration factor was 20 to 40 and 800 ml could be concentrated in 24 hours No initial concentration was needed if the haemagglutination titre was 1/64 or higher The supernatant was treated with 0 03 M EDTA for one hour at +4° C The material was layered on a sucrose cushion which was prepared in two layers The first layer was 2 ml of 60 per cent sucrose in TEN buffer and the upper layer 4 ml of 20 per cent sucrose in TEN buffer The tubes were centrifuged with a SW 25 1 rotor in a Spinco model L 50 ultracentrifuge at 20,000 rpm for 120 minutes The interphase between the 60 per cent and 20 per cent sucrose layers was collected by piercing the bottom of the tube with a hypodermic needle The material was diluted 1 1 with TEN buffer, sonicated for 2 minutes in a Raytheon sonic oscillator model DF 101 in a thin walled centrifuge tube, and layered on a linear 20 to 60 per cent sucrose gradient in TEN buffer The tubes were centrifuged in a SW 25 1 rotor at 22,500 rpm for 150 minutes or 18 hours and the material collected dropwise through the bottom in small fractions These were tested for rubella haemagglutination and the pooled peak fractions of about 1–2 ml were regarded as purified rubella virus The virus was stored at 70° C for not more than 2 months before further analysis

Preparation of radioactive labelled virus Precursors of RNA and proteins labelled with radioisotope were obtained from the Radiochemical Centre Amersham England Labelled virus was produced by the same procedure as unlabelled virus with the following exceptions To produce RNA labelled virus 1 2 μCi/ml uridine-5-³H specific activity 25 Ci/mmol, was added To prepare leucine labelled virus, one μCi/ml of D, L-Leucine-4-5-³H, specific activity 15 8 Ci/mmol, was added to cultures lacking unlabelled leucine Amino-acid-C14(U) mixture from Chlorella protein hydrolysate, specific activity 52 mCi/m-atom of carbon, was added to cultures having only 10 or 20 per cent of the usual amounts of amino acids In all cases the radioactive isotope was added 6 to 18 hours after adsorption of viruses onto the cells

The counting of the specimens was performed with liquid scintillation techniques, using a Packard Tri Carb scintillation spectrometer model 3375 The counts were performed in toluene based scintillation fluids Acid insoluble counts were usually performed, but sometimes the specimens were only dried on filter paper and counted without any other treatment essentially the same results being obtained as with acid precipitable counts

Preparation of soluble antigen from rubella infected cells The rubella infected BHK 21/13S or BHK 21/WI 2 cells were washed three times in PBS and then diluted for 10 per cent (v/v) suspension This was frozen and thawed once or twice NP40 was added to give a final concentration of 0 2 per cent The material was kept at room temperature for one to two hours and shaken frequently The suspension was sonicated for 5 minutes in Raytheon sonic oscillator The insoluble material was removed by centrifugation at 1000 g for 15 minutes and by subsequent centrifugation in a Spinco model L 50, using rotor no 50 at 100 000 g for one hour The supernatant was concentrated 5 to 10 times by forced dialysis against polyethylene glycol 20,000 and dialysed against PBS or TEN buffer The material was stored at —15° C and sonicated for 2 minutes before use

Gradient Centrifugations

All the ultracentrifugations were performed in Spinco models L50 or L2-65B The rotors SW 25 1, SW 39 SW 40 and SW 56 were used The preformed gradients were prepared in a device described by *Ayad* et al (4) Detergents were added to the gradients as indicated in the results The tubes were pierced with a hypodermic needle and the fractions collected in glass tubes or in cups of disposable microtitre U plates An Abbé refractometer was used to measure the refractive index of the fractions The density was read from a curve prepared by weighing 100 μl amounts of CsCl solution of known refractive indexes For sedimentation coefficient determinations bovine serum albumin was used as marker Sedimentation coefficients were calculated according to *Martin et al* (18)

Tests to Detect Rubella Antigens

Complement fixation test (CF) has been described in detail elsewhere (20) For antigen titration four complement fixing units of antisera were used in the presence of two full units of complement The results are expressed as reciprocals throughout

Haemagglutination test (HA) was performed as described by *Halonen et al* (8) The HA results are expressed throughout as reciprocals of HA titres/0 05 ml

Gel precipitation test (GP) is described in detail earlier (20) The only variation in this work was the use of agarose (L Industrie Biologique Francaise, Gennevilliers, France) instead of agar Undiluted rubella antiserum specimens were used in GP tests to detect rubella GP antigens

Antisera A human serum taken 21 weeks after rubella rash was used throughout the study The development of rubella antibodies in that case is reported elsewhere (20) The rubella antibody titre measured by haemagglutination inhibition (HI) was 1/320 and by CF test 1/16, in the GP test the serum produced rubella specific lines against two viral components All tests for rubella antigens were performed with this serum and a serum from a rabbit which had received four intraveneous injections of purified rubella virus at two weeks intervals The total HA units/injection was 12800 The rabbit was bled one week after the last injection The serum had a rubella HI titre of 1/640 and a CF titre of 1/32 and it produced two GP lines against alkaline extracted rubella antigen and no lines against BHK 21 control cells or bovine serum (Fig 1)

Antiserum to BHK 21 cells and bovine serum were prepared in rabbits Washed BHK 21/13S cells were disrupted by sonication and the debris spun down at 1000 g for 15 minutes The cell supernatant or foetal calf serum was mixed with Freund s complete adjuvant and 2 ml of these mixtures were injected subcutaneously into rabbits The injection was repeated three times at three-week intervals and rabbits were bled one week after the last injection The sera were tested in gel precipitation tests in which they were found to contain antibodies against multiple components of BHK cells and bovine serum respectively

Treatment of virus The purified virus was dialysed against PBS or TEN buffer The detergents used were in 2 per cent 5 per cent or 10 per cent stock solutions in PBS or TEN These solutions were added to the virus suspension to give the final concentration required The material was kept at 0° C for 15 or at +37° C for 30 minutes before further analysis if not otherwise stated in the results Treatment with Tween 80 and ether was performed according to the method described elsewhere (9)

RESULTS

Demonstration of structural antigens of rubella virus reacting in gel precipitation Three rubella gel precipitation antigens in alkaline extracted material from rubella infected BHK 21/13S cells have been demonstrated previously These antigens remained in the supernatant after centrifugation at 100 000 g for three hours This type of centrifuged alkaline extracted antigen was used as reference throughout the study

The rubella HA titres of unconcentrated supernatants were 4 to 8 (BHK 21/13S cells) or 64 to 256 (BHK 21/WI 2 cells) and the HA titres of purified concentrated rubella virus preparations were 1024 to 4096

The sedimentation properties and buoyant density of the virus was as described earlier (24, 28)

No reaction was seen when purified virus was tested in GP tests with rabbit hyperimmune serum against BHK cells or bovine serum The purification procedure separated virions from soluble rubella specific material, which was seen in GP tests The GP line produced by purified virus was seen in the antigen well, whereas lines produced by the soluble antigens were usually located in the middle between the antigen well and the antiserum well (Figs 1 and 2) In the latter case the GP lines could be located near the antigen well if the concentrations of the antigens were low The location of the virion line near the antigen well was not due to the low concentration This was confirmed by disrupting the virus by treatment with NP40 The results of GP tests before and after this treatment can be seen in Fig 2 As can be seen at least two lines were produced after treatment and these were located in the middle between the antigen and the antiserum wells

When undisrupted virus was tested on the same plate as the reference soluble antigen for gel precipitation the lines produced by rubella virus and by soluble rubella antigen were found to coalesce in several tests indicating a reaction of identity Treatment of

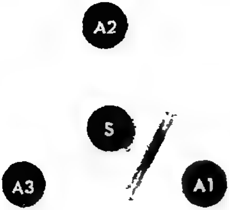

Fig 1 Gel precipitation test of a rubella antiserum prepared in a rabbit by repeated intravenous in ject ons of purified rubella virus
A¹ = rubella virusantigen
A = BHK 21 cell antigen
A³ = bovine serum.
S *rubella antiserum*

TABLE 1 *Effect of Chemical and Physical Treatments on Purified Rubella Virus*

Treatment	Effect on HA titre	Release of b antigen (GP)
Nonidet P40, 0 5 %	4 to 8 fold decrease	+[1]
Tween 20, 0 5 %	none or slight decrease	+
Tween 40, 0 5 %	none or slight decrease	+
Tween 80, 0 5 %	none	+
Tween 60, 0 5 %	none	+
Tween 21, 0 5 %	none	±[2]
Tween 85, 0 5 %	none	—[3]
Tween 65 0 5 %	none	—
Tween 81, 0 5 %	none	—
Tween 61, 0 5 %	none	—
Span 20, 0 5 %	none	—
Tween 80 + ether	2 to 8 fold increase	+
Ether	none	±
DOC, 0 5 %	2 to 4 fold-decrease	+
SDS, 1 %	total disappearance	+
2 ME	total disappearence	±
SDS + 2 ME	total disappearence	±
Periodate, 0 1 M	none	—
+ 37°, 18 hrs	none	—
+ 56°, 30 min	2 to 8 fold increase	—

1 + = soluble GP antigen released
2 ± = soluble GP antigen released, but the reaction very faint
3 — = no release of soluble GP antigen

virus at +37° C for 30 minutes with 0 2 per cent NP40 clearly released an antigen coalescing with a line produced by a soluble antigen (Fig 3) The line produced could be shown to be identical with the b line de scribed earlier (20) The intensity of that line increased when the HA titre of the virus was increased When very high titered rubella virus (HA titre 4096 or more) supplied by Dr *A Vaheri* Department of Virology, University of Helsinki Finland was used, two or three GP lines were seen

Effect of Chemical and Physical Treatment on Rubella Virus

As shown above, a small GP antigen can be released from rubella virus preparations by NP40 treatment A rubella HA titre of 1024 was required to produce a visible GP reaction In the following tests, batches of virus having HA titres of 1024 to 4096 were employed In NP40 treatment a final concentra tion of 0 05 per cent was able to release the maximum amount of GP antigen, but con-

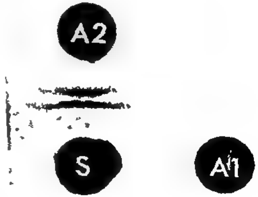

Fig 2 Effect of Nonidet P40 treatment on purified rubella virus Results of a gel precipitation test before and after treatment
A¹ = purified rubella virus
A² = purified rubella virus, treated with Nonidet P40
S = rubella antiserum

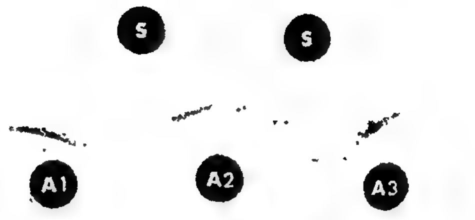

Fig 3 Demonstration of the reaction of identity between antigen released from purified rubella virus and a soluble antigen in rubella infected BHK 21 cells

A¹ and A² = two lots of purified rubella virus treated with Nonidet P40

A³ = alkaline extracted soluble antigen from rubella infected BHK 21/13S cells

S = rubella antiserum

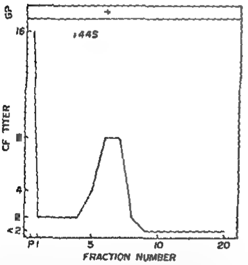

Fig 4 Complement fixation (CF) and gel precipitation (GP) *antigens of rubella recovered from* a 5–20 per cent sucrose gradient after centrifugation of purified *Tween 20 treated rubella virus at* 39,000 rpm for 40 hours in Spinco rotor SW 39
P = *pelleted material*
Fractions 1 to 20 are from the bottom to the meniscus

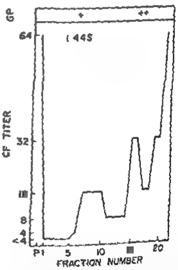

Fig 5 Complement fixation (CF) and gel precipitation (GP) activities *of rubella recovered from* a 5–20 per cent sucrose gradient after centrifugation of purified Nonidet *P40 treated rubella virus* at 39 000 rpm for 40 hours in *Spinco rotor* SW 39
P = pelleted material
Fractions 1 to 21 are from the bottom to the meniscus

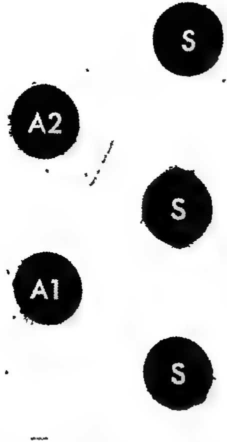

Fig 6 Gel precipitation test to demonstrate the reaction of identity between antigens from rubella virus sedimenting at 13 S and at 3 5 S
A[1] = rubella antigen sedimenting at 13 S
A[2] = rubella antigen sedimenting at 3 5 S
S rubella antiserum

centrations of up to 2 per cent did not have any harmful effect on the antigenicity as tested in GP The treatment with NP40 was usually performed at 37° C or at 20° C but the effect was similar when performed at +0° C for 15 minutes

The effect of some other treatments on HA titres and the release of the gel precipitation b antigen (20) was studied The b antigen was considered to be released when the position of the line was the same as that seen after NP40 treatment The results are shown in Table 1 The treatments with detergents were performed at +37° C for 30 minutes As can be seen the b-antigen was also released from virus by Tween 20, Tween 40, Tween 60 and Tween 80 The HA activity remained at the same level after these treatments Some other treatments produced only slight GP activity but the HA activity was lowered or disappeared totally at the same time Even after treatment with 1 per cent SDS and 1 per cent 2-mercaptoethanol (2-ME) some GP activity could be found if the original HA titre of rubella virus was 2048 or more The finding that some treatments, e g with Tween 80, are capable of releasing b antigen without significantly effecting the HA activity of the material is of interest

Gradient Centrifugation of NP40 and Tween 20 Treated Rubella Virus

Purified rubella virus was treated with NP40 or Tween 20 (T20) and the products were centrifuged through a 5–20 per cent linear sucrose gradient (w/v) which contained 0 05 per cent of the corresponding detergent

T20 treatment released a GP active component which sedimented at about 3 5 S (Fig 4) Some CF activity could be found in the

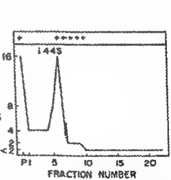

Fig 7 Complement fixation (CF) and gel precipitation (GP) activities in fractions after centrifugation of soluble antigens from rubella infected BHK 21/13S cells in a 5 20 per cent sucrose gradient at 39 000 rpm for 40 hours in Spinco rotor SW 39 Antigen treated with Nonidet P40 and the gradient containing 0 05 per cent of this detergent
P – pelleted material
Fractions 1 to 22 are from the bottom to the meniscus

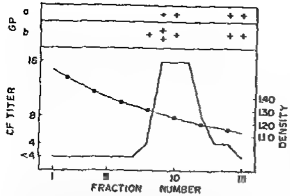

Fig 8 Complement fixation (CF) and gel precipitation (GP) activities after equilibrium centrifugation in CsCl of Nonidet P40 treated purified rubella virus. The centrifugation was performed at 50,000 rpm for 72 hours in Spinco rotor SW 56

same area, but its distribution was wider. No GP activity was detected in the pellet, but in the CF test more activity was found in the bottom than in the middle of the gradients. After treatment of virus with NP40 a similar pattern was found on centrifugation. If the HA activity of the original virus was at least 4096, other antigenic properties of rubella could be detected (Fig 5). A second area of GP activity was found to sediment at 1.0 to 1.3 S. In a GP test this activity was proved to be identical with the activity sedimenting at 3.5 S (Fig 6). The S value of the main GP activity was calculated to be 3.5±0.3 (mean and standard deviation of 5 determinations). A small part of the CF activity was found in the same fractions as the GP activity. It represented *10–30 per cent of the total CF* activity found in the gradients. The main CF activity was found in the pellet and in some gradients in the top fractions (Fig 5). No GP activity was demonstrated in the pellet or in the top fractions although the material was tested undiluted, diluted and concentrated. After NP40 treatment ^{3}H-leucine labelled virus was centrifuged through a similar gradient, in order to demonstrate possible viral components which might not have been detected by the serological techniques used. No additional components were found, and the main activities were in the pellet and in the area around 3.5 S. The latter represented 30–50 per cent of the total ^{3}H-leucine counts. Small amounts of radioactivity was seen in the top fractions.

To compare the GP antigen released from purified virus with the soluble GP antigens from rubella-infected cells, the latter type of antigen was centrifuged through a similar 5–20 per cent sucrose gradient containing NP40. A part of this antigen seemed to be about the same size as the antigen from the virus itself. As can be seen in Fig 7, this cell derived b antigen sedimented in a broader peak (2.8 to 3.8 S) than the antigen from the virus. An identical GP antigen was found this time in the pellet, as indicated by the reaction of identity in a GP test. The CF activities were found in the same fractions as the GP activity.

Purified rubella virus was treated with NP40 and mixed with saturated CsCl in PBS to give an initial density of 1.29 g/ml. The tubes were centrifuged in SW56 rotor at 50,000 rpm for 48 to 72 hours. The results of one centrifugation are shown in Fig 8. Two GP lines were found in some of the fractions, the stronger representing the GP antigen b. The GP activity was distributed in a wide area of densities between 1.25 and 1.30 g/ml. Identical activities could be found *in the top* fractions which represented densities as low

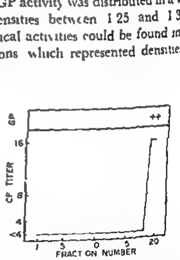

Fig 9 Complement fixation (*CF*) and gel precipitation (GP) activities in *fractions* after centrifugation of NP40 treated *rubella* virus in a 10–40 per cent linear sucrose gradient at 35,000 *rpm* for 1 hour in SW 39 rotor.
Fractions 1 to 20 are from the bottom to the meniscus

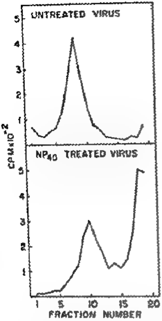

Fig 10 Radioactivity (CPM) in fractions after centrifugation of ^{3}H leucine-labelled virus in 10–40 per cent sucrose gradient at 35 000 rpm for 1 hour in Spinco rotor SW 39 The upper figure shows the results without Nonidet P40 treatment and the lower figure the results of a simultaneous centrifugation with Nonidet P40
Fractions 1 to 20 are from the bottom to the meniscus

as or lower than 1.10 g/ml The main CF activity was found in the same fractions as the GP activities in the middle of the gradients but the top fractions contained only small amounts of CF activity

Demonstration of a Gel Precipitation Antigen as a Structural Component of the Rubella Envelope

Purified rubella virus was treated with 0.1 to 0.2 per cent NP40 for 15 minutes at +0° C and the material was centrifuged immediately afterwards at 35 000 rpm for one hour through a 10–40 per cent linear sucrose gradient in TEN buffer containing 0.05 per cent NP40 In these conditions the CF and GP activities were demonstrated only in the top fractions (Fig 9) In some gradients trace amount of HA activity appeared in the top fractions There was also evidence that fractions in the middle of the gradients contained some CF activity. In these experiments the HA titre of the untreated virus was only 1024 to 4096, so it was possible that the tests were not sufficiently sensitive for detection of faint antigenic activities in the middle of the gradients To test this possibility, ^{3}H–uridine labelled purified virus material was centrifuged through a similar gradient after NP40 treatment When the virus was concentrated and purified from the BHK-21/13S supernatant the uridine label could be found only in the top fractions and in the pellet But rubella virus grown in BHK–21/W1–2 cells and labelled with ^{3}H–leucine split into two components in this type of gradient as shown in Fig 10 The GP activity *b* was found in the top fractions as well as all of the CF activity No CF or GP activity was detectable in the middle of these gradients The S value of the peak GP and CF active material was proved to be about 3.5 S The S value of the material from the middle of the gradient, possibly representing the core of the rubella virus, was between 100 and 200 S A variable amount of the total leucine–^{3}H counts was found in the middle of the gradients, usually 30 to 40 per cent of leucine labelled protein being in the core fractions When ^{3}H uridine labelled rubella virus was split in a similar gradient only about 5–10 per cent of the counts could be found in the top fractions, where the GP activity was situated, and most of the activities were in the middle and at the bottom of the tube

The top fractions were centrifuged to equilibrium in CsCl gradients The CF and GP activities were found in fractions of densities 1.28 to 1.30 g/ml When the total material from ^{3}H-leucine labelled NP40 disrupted rubella virus was centrifuged in similar conditions, the activities were of about the same densities as the material from the top fractions

When radioactive material from the middle of a 10–40 per cent sucrose gradient was centrifuged in a CsCl gradient without any pretreatment material from ^{3}H uridine label

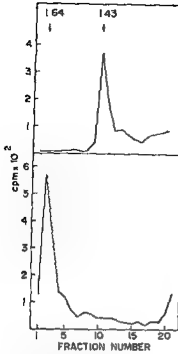

Fig 11 Radioactivity (cpm) in fractions after equilibrium centrifugation in CsCl of core components of ^{3}H uridine labelled rubella virus at 50 000 rpm for 72 hours in Spinco rotor SW 56. The lower figure shows the results without glutaraldehyde treatment and the upper one with this treatment

led virus was found at a density of ca 1 64 g/ml Tritium from double labelled virus (^{3}H-uridine, C^{14} amino acids) was seen in the same area but the C^{14} counts were in fractions of density about 1 30 g/ml If the core material was pretreated with glutaraldehyde (5) immediately after the first centrifugation, and centrifuged in CsCl, the material was found to equilibrate in densities of about 1 43 g/ml to 1 45 g/ml (Fig 11), mean with standard deviation of six centrifugations was 1 44±0 04 g/ml

DISCUSSION

The present study has shown that two or three gel precipitating antigens can be extracted from purified rubella virus The strongest of these antigens was further characterized and found to be identical with the earlier characterized b antigen from rubella infected cells (20,21) Theoretically, it is possible that this antigen is not a structural part of the rubella virus but only associated with the purified virus However, in this study the GP activity became stronger as the HA activity increased This supports the idea that GP antigen really is part of the rubella virus structures, because the HA activity has been shown to be a component of the rubella virion (28) Furthermore, no antigens diffusing as far as the soluble antigens were demonstrated in the GP tests on the purified virus material before detergent treatment *Schmidt et al* (22) suggested that a soluble GP antigen may be part of the rubella vi[rus] structure itself The soluble rubella GP an[ti]gen theta described by *LeBouvier* (14) m[ay] be related to rubella haemagglutinin (15) A small-sized antigen similar to the one d[e]monstrated in this study was extracted fro[m] the rubella virus and detected by platelet ag[]gregation and complement fixation tech[]niques by *Vaheri et al* (30) In some of th[e] arboviruses which resemble rubella small sized antigens have been extracted by treat[]ment with detergents (11)

A nonionic detergent, Nonidet P40 (Shell) was useful in disrupting rubella virus into GP active components This detergent was earlier used to disrupt vaccinia virus in controlled conditions (7) Later it was adapted for use with arboviruses (2) *Vaheri et al* (30) found that rubella virus could be disrupted by NP40 without any appreciable effect on HA activity In this study the HA titre was lowered This effect may be due to the fact that no urea was added to the reaction mixture to increase the stability of rubella haemagglutinin (25)

The GP antigen studied here was small sized having an S value of about 3 5 S In all of the material studied some CF activity was found in the same fractions as the GP activity, but fractions were always found in the gradients with CF but no GP activity This cannot be due to the general sensitivity limit of the GP method because of the high CF titre of the nonprecipitable rubella antigen

This finding is quite striking because the same antisera were used in both tests It may depend on differences in antigens or antibodies The antigen molecules may be of large size or special shape or they may be covered by detergent molecules so that no GP reaction can occur, although the CF reaction is still possible Some antibodies may be nonprecipitating (6) This is especially the case if only a few antigenic determinants are responsible for the production of antibodies The finding by *Vaheri et al* (30) that the rubella platelet aggregation antigen has an S value of about 3 5 S suggests that the same rubella antigen may have complement fixation, gel precipitation and platelet aggregation activities These activities were all found in the same fractions after sucrose gradient centrifugations (29)

The GP antigen studied here seems to be located in the envelope of the rubella virus Using radioactive label it was possible to release this antigen and still have a faster-sedimenting part of the virus It is possible that the latter part is the core (1) of the virus and that it may have CF activity This activity could not be shown in these experiments Under conditions resembling those in this study *Vaheri et al* (30) presented some evidence that it was possible to find rubella core components having S values of about 150 S This component had faint platelet aggregation and complement fixation activities The core fractions of some arboviruses have been shown to have properties similar to those reported here Kaariainen et al (11) found that the core fraction of Semliki forest virus had a density of about 1 40 g/ml *Acheson et al* (1) purified Semliki forest virus core particles from infected chick embroy cells and were able to show that these had a density of 1 47 g/ml in cesium chloride The density of the rubella core in this study seemed to be at the same level pointing to a relatively high content of RNA in these particles It is possible that some protein of core origin has been liberated during the treatments This is reasonable when it is remembered that the core in this study was very labile A similar observation was made by *Holt et al* (10) At least in some experiments undisrupted core fractions have been obtained, because a notable proportion of the total radioactive counts was found in the core fractions In the study of *Kääriäinen et al* (11) on Semliki forest virus only 13 per cent of the total phenylalanine counts was incorporated in the core fraction but 31 per cent of the lysine counts were found in the core (12) There is a possibility that glutaraldehyde treatment may have changed the density of the core particles This could not be proved directly, but in the case of undisrupted rubella virus the density of the unfixed virus seemed to be at about the same level as that of the glutaraldehyde-fixed virus (unpublished observations), which tells against this assumption The "core" fractions in this study contained both RNA and proteins as can be seen from the equilibrium centrifugations Tritiated uridine was found at a density of 1 64 g/ml and proteins at about 1 30 g/ml, which are the density values of rubella RNA (10) and virus specific proteins (30)

The present results indicate that two or three gel precipitation antigens can be found in purified rubella virus preparations One of these was further characterized The characterization of the other structural rubella GP antigens was not possible with the concentrations of rubella virus available

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PURIFICATION OF A SOLUBLE GEL PRECIPITATING ANTIGEN OF RUBELLA VIRUS AND ANTIBODY RESPONSES TO THE PURIFIED ANTIGEN

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A gel precipitating (GP) antigen was purified from rubella infected BHK 21 cells The purification procedure included DEAE cellulose chromatography and ultracentrifugations The purified antigen also had CF antigen activity which represented about 10 per cent of the original amount of CF antigen in infected BHK cells This GP antigen sedimented at about 3 5 S in sucrose gradient centrifugations and equilibrated at about 1 30 g/ml in CsCl gradient centrifugations Immunization of guinea pigs with this antigen resulted in the production of rubella GP antibodies as well as HI and CF antibodies to purified rubella virus The gel precipitating antibody response was measured with this purified antigen After natural rubella infection a slow increase in GP titres was found until the end of the observation period which covered 3 to 4 months A tendency to slow development of rubella GP antibodies was observed in rubella vaccinees Animal immunizations confirmed the slow increase of GP antibodies. Rabbits responded better than guinea pigs when measured by GP, although the latter produced high rubella HI antibody titres

Characteristics of a structural rubella antigen reacting in gel precipitation were described in the previous report (17) This antigen proved to be part of the rubella envelope Partial purification was achieved with the techniques used but the relatively small amounts of purified rubella virus did not allow a more detailed study of this antigen Larger amounts of rubella gel precipitation antigen could be extracted from rubella infected BHK 21 cells With these as starting material a structural gel precipitating antigen of rubella virus was further purified This purification procedure will be described in the present report and the results of determinations of gel precipitating antibodies in sera after natural and experimental rubella infections and after rubella vaccinations are presented

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MATERIALS AND METHODS

Virus and cells The rubella virus was the RA 27/3 strain (25) and BHK 21 cell clones 13S and WI 2 were employed (24) The cell culture techniques have been described earlier (15 16 17)

Preparation of soluble rubella antigen BHK 21 cells in suspension (13S) or roller (WI 2) cultures were infected at multiplicity of infection of one plaque forming unit per cell and maintained as described elsewhere (15 17) The cultures were harvested after 2 to 3 days incubation at +35°C Cells and supernatant were separated by low speed centrifugation The cells were washed three times

in phosphatase buffered saline (PPS) without Ca^{++} and Mg^{++}, pH 7 4, and a 10 per cent cell suspension was prepared in this buffer If the initial HA titre of the supernatant was 1/4 or higher, the virus was harvested from the supernatant by precipitation with 50 per cent saturation of ammonium sulphate The precipitate was dissolved in PBS and the material dialysed against PBS for several days with frequent changes of the dialysing buffer The precipitated material was added to the washed rubella infected cells A nonionic detergent, Nonidet P40 was added to a final concentration of 0 2 per cent and the mixture was kept at room temperature for 1 to 2 hours The suspension was sonicated in a Raytheon sonic oscillator, model DF101, until about 95 per cent of the cells were disrupted as estimated visually under a phase contrast microscope The debris was removed by centrifugation in a PR6 International centrifuge at 1000 g for 15 minutes The pellet was discarded and the supernatant centrifuged in a Spinco model L50, using rotor no 50 at 100,000 g for 1 hour The soluble rubella antigen was in the supernatant which was further processed to obtain purified rubella antigen

DEAE cellulose chromatography Anion exchange cellulose DE23 (W & R Balston Ltd, Maidstone, Kent, England) was prepared according to the manufacturer's recommendations The soluble rubella antigen was dialysed against 0 05 M phosphate buffer, pH 6 9 for 2 to 3 days The buffer was changed twice daily A DE23 cellulose column, 10 × 2 cm was equilibrated with 0 05 M phosphate buffer, pH 6 9 and loaded with dialysed soluble rubella antigen extracted from 1 to 2 ml of washed BHK cells The column was eluted with increasing NaCl concentrations (0 to 0 5 M) in the starting buffer

Ultracentrifugations Spinco L50 and L2 65B ultracentrifuges were used throughout Sucrose gradients were prepared with the aid of a constant speed peristaltic pump (2) The other techniques in the centrifugation procedures are reported elsewhere (17)

Serological techniques The rubella complement fixation (CF) and haemagglutination inhibition (HI) techniques have been described earlier (6, 7) and in gel precipitation (GP) tests a micromethod was used (15) To determine the GP titres of antigens a standard rabbit antiserum (17) was used throughout the study Double or triple titrations of antigen were performed and the dilutions tested against the rabbit serum. The last dilution producing visible precipitate was considered to be the titre of the antigen When serum specimens were tested, the antigen was first titrated against the standard rabbit antiserum The last dilution giving the GP reaction was said to contain one unit of antigen Serial duplicate 2 fold dilutions of sera were then tested against an antigen dilution known to contain 2 to 4 units of antigen The GP titre of a serum was the last dilution giving a GP reaction in these conditions

Immunoelectrophoretic techniques The micro method described by Scheidegger (19) was employed

Serum specimens from rubella patients The serum specimens were taken from 37 army trainees with serologically confirmed rubella infection The first serum specimens were taken from these patients as soon as possible after the rash appeared usually 0 to 2 days after the first signs of illness The subsequent specimens were taken 7 to 14 days later and thereafter at 1 to 2 month intervals From 13 of these patients only two specimens were available, but from the remaining 24 patients three to six specimens were taken

Serum specimens from rubella vaccinees Vacci [illegible]

vaccine HPV 77 DK12 was given by *Dr A Vaheri and collaborators* (26) Two to four serum specimens were taken from each vaccinee

Antisera The method of producing antisera to noninfected BHK 21 cells bovine serum and bovine serum albumin has been described elsewhere (17)

Animal immunizations An intravenous inoculation of purified rubella virus was given to five adult rabbits and blood specimens were taken regularly Similarly, purified rubella virus was inoculated intracardially or intraperitoneally into adult guinea pigs which were bled at frequent intervals All the serum specimens were tested for rubella HI and GP antibodies A group of four guinea pigs was inoculated with a further intracardiac booster of rubella virus and blood specimens were collected at frequent intervals and tested for rubella antibodies

Calculations and statistics The mean titres were calculated by using logarithms of titre values The value 1/10 in HI tests was regarded as 5 the value 1/4 in CF tests as 2 and the value 1/1 in GP tests as 0 5 Student's t test was employed to test the significance of the difference between the mean values

RESULTS

Purification of Rubella GP Antigen from Infected BHK-21 cells

DEAE cellulose (DE23) chromatography proved suitable in the separation of soluble antigens from rubella infected cells When virus preparations were eluted from DE23 columns with a 0 0–0 5 NaCl gradient in 0 05 M phosphate buffer, pH 6 9 all the

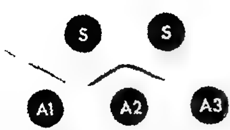

Fig 1 Gel precipitation test showing the reaction of identity between an antigen from purified rubella virus and an antigen from rubella infected BHK 21 cells The latter was partially purified by DEAE cellulose chromatography
A1 = purified rubella virus treated with Nonidet P40
A2 = material from the DEAE chromatography with peak GP activity
A3 = material from the DEAE chromatography with peak CF activity
S = rubella antiserum

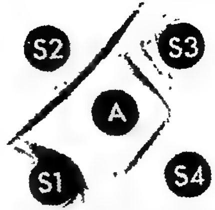

Fig 3 Gel precipitation test on an antigen from rubella infected BHK 21 cells eluted from the DEAE cellulose column with 0 05 M phosphate buffer, pH 6 9
A = material from the DEAE-cellulose column
S1 = antiserum to BHK 21 cells
S2 = antiserum to bovine serum
S3 = rabbit antiserum to rubella
S4 = human antiserum to rubella

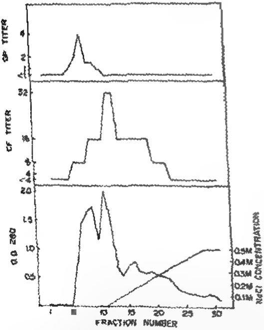

Fig 2 Rubella gel precipitation (GP) and complement fixation (CF) activities, and the optical densities at 280 nm (O D 280) of fractions after DEAE cellulose chromatography Soluble material from rubella infected BHK 21 cells was eluted with a linear (0 to 0 5 M) NaCl gradient in 0 05 M phosphate buffer pH 6 9

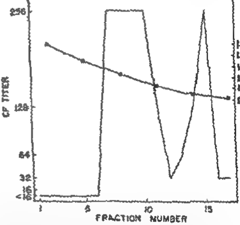

Fig 4 Complement fixation (CF) activity in fractions after equilibrium centrifugation of the peak CF positive material from the DEAE chromatography in CsCl at 50,000 rpm for 72 hours in a Spinco rotor SW 56

detectable GP antigen b was found in the first eluates Most of the CF activity was eluted with higher salt concentrations. The eluted GP antigen was identical with the b-antigen (14) of the rubella envelope (Fig 1) Results of a representative chromatography experiment are shown in Fig 2 A small part

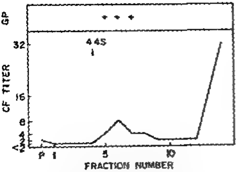

Fig 5 Rubella gel precipitation (GP) and complement fixation (CF) activities in fractions after centrifugation at 39 000 rpm for 40 hours in Spinco rotor SW 39 of material from the peak GP fractions after DEAE cellulose chromatography
P = pelleted material
Fractions from 1 to 15 are from the bottom to the meniscus

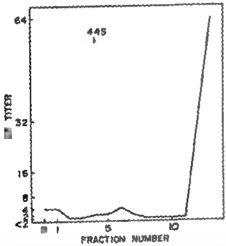

Fig 6 Rubella gel precipitation (GP) and complement fixation (CF) activities in fractions after centrifugation at 39 000 rpm for 40 hours in Spinco rotor SW 39 of material from the peak CF positive fractions after DEAF chromatography
P = pelleted material
Fractions 1 to 13 are from the bottom to the meniscus.

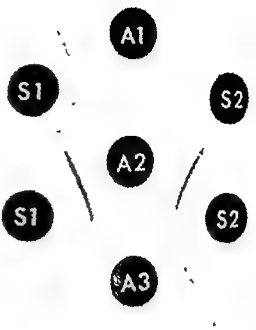

Fig 7 Gel precipitation of 4 fold concentrated material from the CF peak and from the GP peak after DEAE cellulose chromatography against two rubella antiserum specimens
A1 = concentrated antigen from the CF peak of a DEAE cellulose chromatography
A2 and A3 = antigens from the GP peak of a DEAE chromatography
S1 = human antiserum to rubella
S2 = rabbit antiserum to rubella

of the CF activity is eluted in the same fractions as the GP activity, but the main CF activity is found in the later eluates The peak of the CF activity was in fractions with 0 05 to 0 1 M NaCl concentrations, but some activity was seen in fractions up to 0 3 M NaCl concentration

After concentration the eluted rubella gel precipitation antigen was tested against antiserum to BHK-21 cells, to bovine serum and against rubella antisera As can be seen in Fig 3 the eluted GP antigen reacted with each of these antisera

The peak GP and CF fractions were pooled separately and analysed in CsCl gradients The fractions with GP activity banded at a density of about 1 30 g/ml. The CF activity peak from the DE23 chromatography banded in two density areas One of these formed a broad peak at about 1 30 g/ml and the other at 1 13 to 1 15 g/ml (Fig 4) The denser activity represented 60 to 70 per cent of the CF activity found in the gradients and the

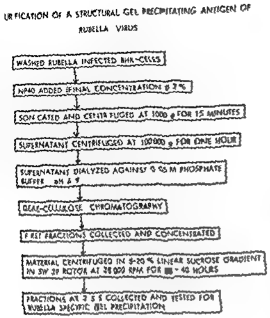

Fig 8 Purification of a gel precipitating structural antigen of rubella virus

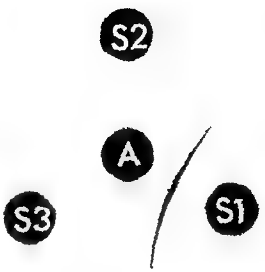

Fig 9 Gel precipitation test of purified rubella gel precipitating antigen against antisera to rubella virus, BHK 21 cells and bovine serum
A = purified rubella antigen
S1 = antiserum to rubella
S2 = antiserum to BHK 21 cells
S3 = antiserum to bovine serum

lighter one had 15 to 20 per cent of the total CF activity Thus the equilibrium density gradient could not clearly separate the gel precipitation activity from the main CF activity

A rate zonal separation through a 5 to 20 per cent linear sucrose gradient was found earlier to be an efficient technique for separation of the GP activity from the main CF activity (17) When DE23 eluates containing GP activity were centrifuged in gradients of this type the GP and a part of the CF activity were always found at 3.5 S (Fig 5) Of the input CF activity, 25 to 55 per cent was usually found in that area The GP/CF titre ratio at 3.5 S was 1/4 to 1/8 When the peak CF fractions from DE23 eluates were centrifuged in a similar gradient, some CF activity was found at 3.5 S area of the gradient but it represented only 5 to 10 per cent of the total CF activity in these gradients The main part of the CF activity was in the top fractions (Fig 6)

The CF peak fractions from DE23 chromatography were tested in various dilutions and concentrations in GP in order to find out whether the lack of GP activity in these fractions was only due to the concentration factor of the antigen No activity was found in the diluted antigen, but in the same antigen concentrated 2- to 4 fold some GP activity against rubella antisera was seen Fig 7 shows the results of this GP test These results indicate that the GP antigen preparation and the main CF antigen preparation from the DE23 chromatography contain rubella antigens which are not identical

On the basis of the observations described above a procedure was designed to purify the gel precipitation antigen b from rubella-infected BHK-21 cells This method [illegible] summarized in Fig [illegible] This procedure was used in the purification of the antigen for subsequent

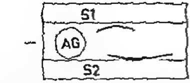

Fig 10 A scheme on the results of *immunoelectrophoresis* The purified rubella antigen was electrophoresed through 1 per cent agarose in 0 09 M phosphate buffer, pH 8 6, at 17 V/cm for 1 5 hours and the precipitates were developed by antisera to bovine serum albumin and to rubella at room temperature for 72 hours
AG = purified rubella antigen
S1 = antiserum to rubella
S2 = antiserum to bovine serum albumin

Fig 11 Gel precipitation test of a guinea pig anti rubella antiserum against rubella antigen and bovine albumin before and after adsorption of serum with aggregated bovine serum
A1 = antigen from rubella infected BHK 21 cells
A2 = bovine albumin
S1 = guinea pig antiserum to a rubella antigen unadsorbed
S2 = guinea pig antiserum to a rubella antigen adsorbed
S3 = rabbit antiserum to rubella

GP tests with rubella antisera The recovery of the antigen was 5 to 10 per cent of the original activity

Tests for purity of the GP antigen The purified rubella GP antigen was tested in gel precipitation against antisera to BHK 21/13S cells, bovine serum, bovine serum albumin, or rubella antiserum Only one precipitation line was observed, and this was specific for rubella (Fig 9) In some lots of antigens a reaction against antiserum to bovine serum was observed This contaminating antigen of bovine origin was proved to be bovine albumin in gel precipitation tests using the specific antiserum In immunoelectrophoresis the bovine serum line could be separated from the rubella specific line (Fig 10) which migrated only a small distance in electrophoresis

As a further test of the purity of the antigen, four guinea pigs were immunized with a purified rubella GP antigen reacting only with antiserum to rubella Two subcutaneous inoculations in Freund's complete adjuvant were given at three week intervals and after another 3 weeks an intravenous injection The blood was collected one week after the last injection In Table I are shown the results of antibody tests As can be seen all of the animals developed antibodies to rubella and bovine serum albumin The immunoglobulins to bovine serum albumin could be absorbed with an immunoaggregate prepared from bovine serum, using the glutaraldehyde method described by Avrameas and his collaborators (1) The results of a GP test before and after adsorption can be seen in Fig 11 The antibody activity in these adsorbed sera against rubella b antigen was too weak to be suitable for practical use in gel precipitation tests The guinea pig sera did not react with BHK 21 control antigen in GP tests (Table I) indicating that the immunizing antigen had not contained any immunogenic substance from BHK 21 cells It was of interest to discover that HI and CF antibodies to purified rubella virus were developed in these guinea pigs However no CF antibodies were detected with alkaline extracted rubella antigens

The CF and GP activities were measured at various steps of the purification procedure Only DEAE cellulose chromatography and

TABLE 1 *Antibodies to Rubella Bovine Albumin and BHK Cells in Sera from Guinea Pigs after Immunization with a Partially Purified Gel Precipitating Antigen of Rubella*

Guinea pig no	Rubella titres		Gel precipitation		
	HI	CF (vial)	Rubella	Bovine albumin	BHK antigen
18	80	32	+	+	—
19	160	64	+	+	—
22	10	16	+	+	—
23	160	16	+	+	—

the subsequent gradient centrifugation are capable of purifying the GP antigen from most of the rubella CF activity Only 0 6 per cent of the original CF activity was found in the GP positive fractions, where 5 per cent of the original precipitation activity was detected This means that about 12 5 per cent of the original CF activity is connected with the GP antigen b

The above results indicate that, with the procedure described, a rubella gel precipitating antigen can be purified from the rubella infected BHK 21 cells, but that bovine albumin remains in some preparations as a contaminant Provided this is kept in mind, the antigen can be used to measure the antibody response after natural and experimental rubella infections

Antibodies to rubella GP antigen b after natural rubella infection Serum specimens from 37 army trainees were tested for rubella HI, CF and GP antibodies The antigen in the CF tests was alkaline extracted antigen from the cells and the antigen in the GP tests was purified antigen b

Almost all of the patients had HI antibodies to rubella at the time of appearance of the rash The CF antibodies were detectable some days later and, in specimens taken 2 to 3 weeks after the onset of the rubella infection CF antibodies were always present During the first week after the rubella rash appeared only a few specimens reacted in the rubella GP test About 50 per cent of the patients had converted to GP positive in 3 weeks but only after 5 to 6 weeks were all of the serum specimens positive in GP tests

The serum specimens were grouped according to the time after the onset of the illness There was only one serum specimen from each of the patients in each group The geometric mean titres were calculated for rubella HI CF and GP in each period after infection The results can be seen in Fig 12 The mean HI titre is already elevated at 0 to

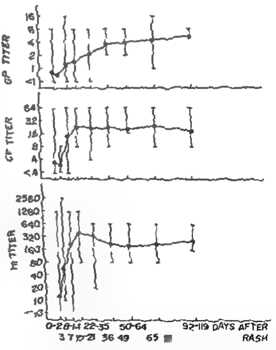

Fig 12 Geometric mean, and range of titres in rubella haemagglutination inhibition (HI), complement fixation (CF) and gel precipitation (GP) tests on serum specimens from 37 army *trainees* after rubella infection The CF antigen was crude alkaline extraction antigen from cells and the GP antigen was purified b antigen from the cells

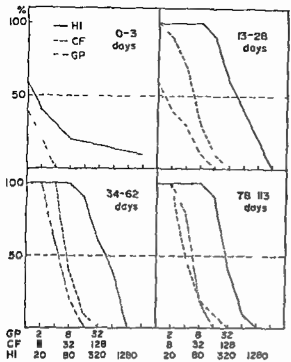

Fig 13 Immunological profiles in rubella haemagglutination inhibition (HI), complement fixation (CF) and gel precipitation (GP) tests on specimens from 10 patients Specimens were taken at indicated times after the rash appeared and the curves represent the cumulative percentages of specimens at various titre levels

2 days after the beginning of the disease and it reaches a peak in 15 to 21 days A slight fall in the mean titre is seen later and a tendency to higher titres ■ observed in the last specimens This rise is not statistically significant In the first 7 days after the appearance of the rubella rash, no clear increase in mean CF titres is demonstrable, but the main increase ■ seen in the second week The peak CF titre ■ reached at the same time as the peak HI titre

In the GP test ■ slight increase in mean titres was seen in the second week The mean GP titre reached only 25 per cent of its highest value when the HI and CF titre peaks were seen The mean GP titre increased during the observation period, and a significant ($p<0\,005$) increase in mean titre was shown after 22 to 35 days

From 10 patients specimens taken 0 to 3, 13 to 28, 34 to 62 and 78 to 113 days after infections were available Immunological profiles of the tests (13) are seen in Fig 13 The curves represent cumulative percentages of specimens at various titre levels The mean titres were calculated from this figure These mean values are shown in Table 2 The results were similar to those for the total material The GP titres had a tendency to increase until the end of the observation period

Antibodies to rubella GP antigen b after rubella vaccination Serum specimens from 9 adult women exposed to rubella vaccination with ■ live attenuated rubella vaccine, HPV 77-DE5, were available Two or three specimens were taken, one before vaccination, the others at 14 to 21 days and 52 to 141 days after vaccination The results of rubella antibody tests are seen in Table 3 All 9 rubella negative patients converted to HI positive, but only 3/9 had detectable rubella CF antibodies in specimens after vaccination In GP tests 5/9 were positive after vaccination If the two patients with the last specimen taken only 14 days after vaccination are excluded 5/7 (about 70 per cent) were positive in the GP test and 2/7 (about 30 per cent) in the CF tests The GP titres were at the same level or higher in specimens taken 52 to 141 days after vaccination than in those taken earlier

TABLE 2 *Mean Titres of Rubella Haemagglutination Inhibition (HI) Complement Fixation (CF) and Gel Precipitation (GP) Titrations of 10 Rubella Cases (From Immunological Profiles)*

Days after appearance of rubella rash	HI	CF	GP
0 3	14	< 4	< 1
13 28	456	23	14
34–62	456	29	48
78 113	279	16	57

Among the 21 vaccinees in the HPV-77 DK12 series 8 had rubella HI antibodies at the time of vaccination and 13 were negative in rubella HI tests One specimen was always available before vaccination and two to three

TABLE 3 *Rubella Haemagglutination Inhibition (HI), Complement Fixation (CF) and Gel Precipitation (GP) Titres after Vaccination with Rubella Vaccine HPV-77/DE 5*
The CF antigen was crude alkaline extract on antigen from the cells and the GP antigen was purified b-antigen from the cells

Patient	Serum taken days after vaccination	HI titres	CF titres	GP titres
K V	0	<10	4	<1
	14	10	4	1
	96	160	16	4
R O	0	<10	4	<1
	15	10	4	1
	98	20	4	1
V L	0	<10	4	<1
	15	10	4	1
	106	40	4	1
M L	0	<10	4	<1
	15	20	8	1
R J	0	<10	4	<1
	15	10	4	<1
P L L	0	<10	4	1
	16	10	4	1
	139	20	4	1
M G H	0	<10	4	1
	21	160	4	1
	141	160	8	2
R T	0	20	4	1
	17	20	4	2
	52	80	4	2
E R	0	20	4	1
	16	20	4	1
	55	80	4	2

TABLE 4 *Seroconversion after Rubella Vaccination with HPV 77/DK12 Vaccine*

I *Patients with low levels of antibodies before vaccination*			
HI test	7/8		88 %
CF test	1/8	=	13 %
GP test	2/8	=	25 %
II *Patients without rubella antibodies before vaccination*			
HI test	13/13		100 %
CF test	3/13		23 %
GP test	8/13		62 %

after vaccination The effect of vaccination was measured with HI CF and GP techniques and the results are shown in Table 4 An interesting finding was that only 25 per cent of the patients converted in the CF test with alkaline extracted antigen but among these about 60 per cent were found to be GP positive after vaccination None of these patients had rubella HI antibodies before vaccination and all of them converted to HI positive

In some cases specimens taken 7 weeks after vaccination were still negative in GP tests although HI conversion had occurred but in specimens taken some months later the GP test was positive This indicates that a higher number of positive GP reactions could have been found if more specimens had been available from these patients In a few cases specimens taken about 15 months after vaccination were available The HI and GP titres were at about the same level as in specimens taken 6 to 8 weeks after vaccination

In most of the specimens the ratio of HI

TABLE 5 *Geometric Means and Range of Titres in Rubella Haemagglutination Inhibition (HI) and Gel Precipitation (GP) Tests on Serum Specimens from five Rabbits after One Intravenous Injection of Rubella Virus*

Test	Serum specimen taken weeks after rubella virus injection						
	0	1	2	3	4	6	8
HI test							
geometric mean	<10	13	53	105	70	26	20
titre range from	<10	<10	20	40	40	10	<10
to	<10	40	320	320	160	80	40
GP test							
geometric mean	<1	0 9	1 8	4 6	7 0	8 0	8 0
titre range from	<1	<1	<1	<1	1	4	4
to	<1	1	4	16	16	16	16

TABLE 6 *Geometric Means and Range of Titres in Rubella Haemagglutination Inhibition (HI) and Gel Precipitation (GP) Tests on Serum Specimens from Four Guinea Pigs after One Intracardiac Injection of Rubella Virus*

Test	Serum specimen taken days after rubella virus injection					
	0	10	13	21	32	54
HI test						
geometric mean	<10	80	320	5120	1520	810
titre range from	<10	10	20	2560	640	640
to	<10	320	2560	10240	5120	1280
GP test						
geometric mean	<1	<1	0 6	0 8	1 7	3 2
titre range from	<1	<1	<1	<1	1	2
to	<1	<1	1	4	8	8

titres to GP titres varied from 10 1 to 40 1, but there were some sera having a HI titre of 1 80 which gave no reaction in rubella GP tests

Antibodies to rubella GP antigen b after immunization of rabbits and guinea pigs To five adults albino rabbits 35,000 haemag glutinating units of purified rubella virus were inoculated, corresponding to about 1 5 to $3\,0 \times 10^{10}$ plaque-forming units (26) The inoculations were given intravenously into the ear veins The specimens were taken at indicated intervals (Table 5) after the inoculations All the rabbits responded to this single inoculation The rise in HI titres was rapid (Table 5) The first significant rises could be shown in specimens taken after one week and the peak of the titres appeared three weeks after inoculation Afterwards a rapid decrease in HI titres was detected By the eighth week one of the animals was already HI-negative The mean HI titre remained at the same level between 8 and 16 weeks The GP titres began to rise later than the HI titres In some specimens taken 2 weeks after inoculation a slight rise in GP titre was seen In three weeks all of the rabbits had converted to GP-positive The highest level of the mean titre was reached at 6 to 8 weeks after inoculation A point of interest was the fact that the GP titres of all the rabbits were at detectable levels in the last specimens taken although some animals were already negative in the HI test The ratio of HI titres to GP titres varied from 80 1 to <10 1

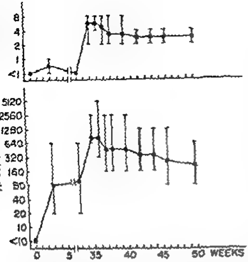

Fig 14 Development of rubella haemagglutination inhibition (HI) and gel precipitating (GP) antibodies in four guinea pigs after two intracardiac inoculations The first injection was given at the time 0 weeks and the second inoculation at 33 weeks

To four adult guinea pigs 2560 haemagglutinating units of purified rubella virus, corresponding to about 1.2 to 2.5×10^9 plaque forming units, was given intracardially Guinea pigs were also inoculated intraperitoneally with a similar dose The animals were bled by intracardiac puncture at frequent intervals and the serum specimens were tested for rubella HI and GP antibodies All guinea pigs, except one that was given rubella virus intraperitoneally converted to rubella positive in the HI test This seroconversion was better in the group that received an intracardiac inoculation than in the group given to intraperitoneal inoculation, the differences in geometric means being 10 to 15 times The results of HI and GP tests on the specimens from the former group are shown in Table 6 The peak HI titres were usually reached in three weeks and a decrease was observed two to three weeks later The GP titres were generally at the highest level two to three weeks later than the HI titres The ratio of HI titres to GP titres varied from 2560:1 to 160:1 This may explain why only two guinea pigs in the group inoculated into the peritoneal cavity converted to GP-positive In the HI test only one animal in this group had titres at the same level as animals in the other group

A group of four guinea pigs was given two intracardiac inoculations of purified rubella The amount of rubella in the two inoculations was 2560 haemagglutinating units and the second inoculation was given 33 weeks after the first The results are seen in Fig 14 The effects of the first injection on the rubella titres are the same as described above The second inoculation resulted in a rapid increase in both HI and GP titres. The HI titres reached the level of 1/320 to 1/2560 and remained at higher levels than before the second inoculation for at least 15 weeks The GP titres reached levels of 1/2 to 1/8 and remained at levels of 1/2 to 1/4 for the remainder of the observation period The HI/GP ratio was between 320:1 and 40:1

DISCUSSION

The results of the present study indicate that a rubella gel precipitating antigen can be extracted and purified from infected BHK-21 cells The same antigen was earlier shown to be part of the rubella envelope (17) and to have CF and PA activities (23) Although such preparations after purification usually showed only specific rubella GP activity, antibodies to bovine serum albumin were also produced in guinea pigs after immunization with this antigen Similarly, the presence of small amounts of other rubella antigens may explain the fact that this immunizing antigen produced rubella-specific HI antibodies in guinea pigs These two rubella antigens (HA and GP) may be of almost the same size, and may to some extent have remained together through the whole purification procedure, although only one antigen was detected in the GP tests The possibility still remains that this rubella GP antigen is part of rubella haemagglutinin The likelihood that this is so is further strengthened by the fact that this antigen seems to be in the envelope of the rubella virus (17)

Some CF activity was always detectable in the purified rubella GP antigen. It could again be a contaminant of the preparations, but other work (27) supports the idea that this antigen carries part of the CF activity in the rubella preparations. This CF activity represented only about 10 per cent of the total CF activity in rubella-infected BHK-21 cells and the main part of the CF activity could be separated from the GP activity. The CF antigens used in diagnostic work (6) do not satisfactorily measure antibodies to the complement-fixing GP antigen studied here, and these antibodies seem not to be measurable with the other conventional techniques.

The main CF activity was found in two physical forms in gradient centrifugations. Part of it banded in CsCl centrifugations at about 1.30 g/ml, but another part of this soluble CF antigen was found at densities of 1.13 to 1.15 g/ml. In rate zonal centrifugations the main CF activity was found in the top fractions. The findings by *Schmidt et al.* (21) also indicated a slowly sedimenting CF antigen. The origin of the main CF activity and its relationship to the rubella virus structure remain to be solved. The possibility still exists that part of the slowly sedimenting CF antigen carries the same rubella protein as the GP antigen, but is associated with lipid material or with the detergent used. Rubella GP antigens of two sizes having the same antigenic determinant were previously found after disruption of rubella virus with Nonidet P40 (17).

It is well known that rubella HI antibodies rise soon after infection and remain at comparatively high levels for years afterwards (7). Similar results were obtained in these series of rubella patients. Antibodies detectable with CF techniques can be seen some days later than HI antibodies (11). The results reported here are in agreement with previous observations. The GP results after rubella infection were different because a continuous increase in mean GP titres was observed throughout the observation period. Our earlier results (15) and the observations by *Le Bouvier* (8, 9) had indicated a slow increase in rubella GP antibodies. It has also been reported that rubella neutralizing antibodies have a tendency to increase for many months after rubella infection (10). It is possible that an antigenic stimulus persists for years in certain cells, probably in lymphocytes, causing a slight increase in antibodies to some parts of the rubella envelope. The results of a two-year follow-up of rubella antibodies by *Tsukan et al.* (29) could support also this idea. It is known that the affinity of antibodies can change. It has been suggested that the elevated rubella neutralization titres seen for many months after a rubella infection (10) may be due to changes in the affinity of the antibodies (22). The same hypothesis may explain the GP results in this work. Although this GP antigen is like the PA antigen of rubella (23), similar slow increase in titres was not found in the latter test (30). Thus, the slow increase in GP titre may depend on some special features of the GP test.

Rubella vaccinations elicited the development of GP antibodies. *Le Bouvier* (8) reported that GP antibodies to the rubella antigens theta and iota could be seen after rubella vaccination when rubella virus vaccine RA27/3 was used, but rubella vaccine strains HPV-77 and Cendehill caused mainly the synthesis of anti-theta antibodies. It was found in this study that GP antibodies were formed more frequently than CF antibodies after vaccinations, although the CF technique is generally more sensitive than the GP technique. This seems to suggest that the main CF activity is different from the GP activity described here and that rises in CF titres after rubella vaccination cannot often be seen (20).

The animal experiments are in agreement with the view of a slow development of GP antibodies to rubella GP antigen. Although rabbit cells are known to be susceptible to rubella infection and adult rabbits can be infected with rubella virus (3, 14), the rise in HI titre was only transitory. On the other hand, the GP titres rose more slowly but remained at comparable high levels for weeks

indicating that the HI and GP antibodies are different Guinea pigs are known to be poor producers of GP antibodies (5) In this study, the primary GP response after one rubella injection was weak although the rubella HI antibody titres were usually very high This is in agreement with earlier observations (18) that rubella can produce infection in quinea pigs A booster injection into guinea pigs seemed to result in higher GP antibody titres These were comparable with the titres in rabbit sera and the antibody titre ratios (HI/GP) were at about the same levels as in rabbits after a single injection Therefore guinea pig antisera may be useful in GP tests if more than one injection of antigens is given

Diagnosis of rubella is often a major problem especially in pregnant women, because the first serum specimens are seldom taken before the time of peak HI and CF titres This problem can sometimes be solved by demonstrating rubella specific IgM antibodies (4) The GP techniques described here may find a practical application to this field because, 3 weeks after rubella infection a rise in rubella GP antibodies can still be seen This application combined with further characterization of the main rubella CF antigen may be worth considering

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EVALUATION OF NEUTROPHIL GRANULOCYTE FUNCTIONS

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Two methods (I and II) for the evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes in human disease are described by which the total number of viable bacteria and the number of viable intracellular bacteria in an in vitro phagocytic system are measured In method I, the number of viable intracellular bacteria was determined using phenylbutazone for the inhibition of intracellular killing of bacteria and antibiotics for the inactivation of extracellular organisms In method II, extracellular and intracellular bacteria were separated by differential centrifugation The results obtained by method II were markedly obscured by contamination with extracellular bacteria Method I provided a more accurate evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes

Several disease syndromes characterized by increased susceptibility to bacterial infections have been related to defects in either phagocytosis or intracellular killing of bacteria by neutrophil granulocytes (3, 4, 8, 9, 10) Diagnosis of these disease syndromes requires analyses of both phagocytic and bactericidal activities of the granulocytes (3, 10) Many of the studies in the past, however have been confined to the phagocytic activity and minor attention has been paid to the dynamics of the intracellular phase (for review see 5) A major problem has remained namely the separation of extracellular and intracellular bacteria in an in vitro phagocytic system in order to evaluate and compare the phagocytic and intracellular bactericidal processes (2 14) The original technique of *Maaloe* (6) using differential centrifugation for the separation of the granulocyte and bacterial populations has been adopted and modified by several investigators (1, 10, 11) These techniques measure cell-associated bacteria and bacteria killed in an *in vitro* phagocytic system, but do not permit separation of phagocytized bacteria from bacteria adhering to the external granulocyte wall Contamination with extracellular bacteria and immune adherence of bacteria to contaminating erythrocytes may also significantly obscure the results (2, 14)

To solve these problems a new method has been developed for the determination of the number of viable intracellular bacteria, using high concentrations of antibiotics for the inactivation of extracellular bacteria and phenylbutazone for the inhibition of intracellular killing of bacteria (13) This method facilitates a precise *in vitro* evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes (12)

In the present study, the results obtained by this new method have been compared to those using differential centrifugation for the separation of extracellular and intracellular bacteria

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MATERIALS AND METHODS

Leucocytes

Heparinized venous blood (10 units of heparin per ml of blood) was obtained from normal individuals and layered on top of a two phase cell separation system in Falcon disposable plastic tubes (16×150 mm) The cell separation mixture contained 10 parts Isopaque (Natrii-N-methyl 3 5-diacetamido-2,4,6-trijodbenzoas) 33 9 per cent (obtained by dilution with distilled water of Isopaque 75 per cent manufactured by Nyegaard & Co , Oslo, Norway) and 20 parts dextran 6 per cent (obtained by dilution with distilled water of Dextran 500 provided by Pharmacia, Uppsala, Sweden) Volumes giving blood columns of 60 to 70 mm were used and the volume of the cell separation mixture was ¾ of the blood volume When the erythrocytes had passed the interface between plasma and Isopaque-dextran, the leucocyte rich plasma layer was withdrawn and centrifuged at 500 g for 5 minutes The cellular pellet was twice washed in 5 ml heparinized saline (1 unit heparin per ml saline) by centrifugation at 500 g for 5 minutes After the final centrifugation, a differential count was made and the cells were resuspended in Hank's balanced salt solution containing 0 1 per cent gelatin to make concentrations of approximately 1×10^7 neutrophil granulocytes per ml Erythrocyte contamination in 10 consecutive specimens varied from 14 to 42 per cent (mean 30 per cent), eosinophil granulocyte contamination from 1 to 3 per cent (mean 2 per cent), basophil granulocyte contamination from 0 to 2 per cent (mean 0 8 per cent), and finally lymphocyte monocyte contamination varied from 10 to 18 per cent (mean 15 per cent) Platelet contamination was minimal and less than 0 02 per cent of the autologous serum remained

Serum

Pooled fresh normal serum from 6 adults was stored in 1 ml aliquots at −30° C Immediately prior to each experiment, 1 ml freshly thawed serum was added to Hank's balanced salt solution containing 0 1 per cent gelatin to make appropriate serum concentrations All experiments in the present study were performed with the same pool of serum

Bacteria

Staphylococcus aureus "Oxford" ("Heatly" strain, obtained from the National Collection of Type Cultures, Colindale London 1958) was cultured overnight in Penassay broth (Difco) and then centrifuged at 1500 g for 10 minutes The bacterial pellet was twice washed in 10 ml 0 45 per cent saline by centrifugation at 1500 g for 10 minutes After the final centrifugation, the bacteria were resuspended in Hank's balanced salt solution to an optical density of 0 6 at 620 nm in a Beckman spectrophotometer This suspension was diluted in Hank's balanced salt solution containing 0 1 per cent gelatin to a concentration of 8 12×10 colony forming units per ml

Antibiotic sensitivity tests for the *Staphylococcus aureus* strain were performed by the tube dilution technique using an inoculum of approximately 1 1 bacteria per ml The minimum inhibitory concentrations were 0 03 units per ml for penicillin G and 0 6 µg per ml for streptomycin

Leucocyte-Bacteria Suspension

0 5 ml leucocyte suspension, 0 1 ml bacteria suspension, and 0 4 ml diluted serum were added in 12×75 mm disposable plastic tubes This provided approximately 2 bacteria per neutrophil granulocyte The final serum concentration in experiment 1 was 10 per cent and in experiment 2 0 2 per cent (vide infra) The tubes were incubated at 37° C with end over end rotation in a Heto rotomixer (manufactured by Heto Birkerod, Denmark) to promote contact between bacteria and leucocytes Samples were removed at prescribed intervals for determinations of the total number of viable bacteria the number of viable intracellular bacteria and viable cell associated bacteria The bactericidal activity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or viable intracellular bacteria provided normal phagocytic activity (12) The number of bacteria phagocytized equals the number of bacteria killed plus the number of viable intracellular bacteria (12)

The Total number of Viable Bacteria

$^1/_{100}$ ml of the leucocyte bacteria suspension was added to 1 ml distilled water to facilitate osmotic disruption of the leucocytes Quantitation of viable bacteria was made from appropriate dilutions of this suspension using a standard pour plate technique and Penassy agar (Difco)

The Number of Viable Intracellular Bacteria

$^1/_{100}$ ml of the leucocyte bacteria suspension and 1 ml Hank's balanced salt solution containing 0 1 per cent gelatin 500 µg streptomycin, 500 units penicillin G and 2 mg phenylbutazone (manufactured by Geigy Basel Switzerland) were added to 12×75 mm disposable plastic tubes, incubated at 37° C for 15 minutes, and centrifuged for 10 minutes at 500 g The cellular pellet was twice washed in 5 ml Hank's balanced salt solution by centrifugation at 500 g for 10 minutes and resuspended in 1 ml distilled water to facilitate osmotic disruption of the leucocytes Quantitation of viable

bacteria was made by the standard pour plate technique (see above)

The Number of Cell Associated Bacteria

$^{1}/_{100}$ ml of the leucocyte bacteria suspension was added to 5 ml Hank's balanced salt solution at 4° C and centrifuged for 10 minutes at 100 g The cellular pellet was twice washed in 5 ml Hank's balanced salt solution by centrifugation at 100 g for 10 minutes and resuspended in 1 ml distilled water for osmotic disruption of the leucocytes to occur Quantitation of viable bacteria was made by the standard pour plate technique (see above)

RESULTS

Experiment 1

The final serum concentration in the leucocyte-bacteria suspension was 10 per cent. During the early phase of incubation, a marked difference between the number of viable cell associated and viable intracellular bacteria was observed, the number of cell-

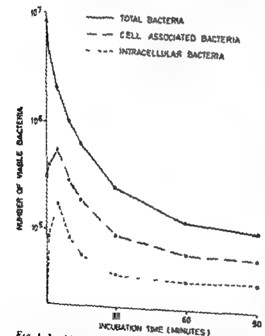

Fig 1 Viable bacterial counts in an *in vitro* phagocytic system of 1 ml containing 5×10^{6} neutrophil granulocytes and 10 per cent serum (mean of five experiments) The number of viable intracellular bacteria is determined by the new method and cell associated bacteria by differential centrifugation

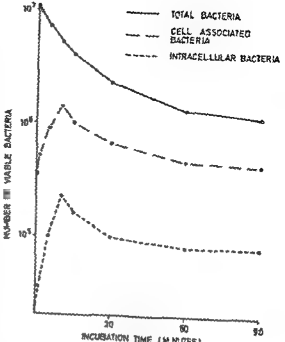

Fig 2 Viable bacterial counts in an *in vitro* phagocytic system of 1 ml containing 5×10^{6} neutrophil granulocytes and 0 2 per cent serum (mean of five experiments) The number of viable intracellular bacteria is determined by the new method and cell-associated bacteria by differential centrifugation

associated bacteria being several times as high as the number of intracellular bacteria (Figure 1) During the later phase of incubation, when the total number of viable bacteria in the leucocyte-bacteria suspension was markedly reduced, the difference was less pronounced, but still the ratio was about 2 1

Experiment 2

The final serum concentration in the leucocyte-bacteria suspension was only 0 2 per cent, reducing significantly the phagocytic activity compared to the test system in experiment 1 with a serum concentration of 10 per cent (12)

During the early phase of incubation, the difference between the number of viable cell-associated bacteria and the number of viable intracellular bacteria was significantly greater than in experiment 1 (Figure 2) Also during the later phase of incubation when the total

number of viable bacteria in the leucocyte-bacteria suspension remained higher than in experiment 1, the ratio of cell-associated bacteria to intracellular bacteria was higher

DISCUSSION

Phagocytosis and *intracellular killing of bacteria* are important physiological functions of neutrophil granulocytes and numerous attempts have been made to relate abnormalities *in these functions* to the development of bacterial infections However, differences in experimental design and difficulties of methodology have resulted in divergent interpretations of experimental results A major problem has remained, namely the elimination of nonphagocytized bacteria in an *in vitro* phagocytic system after a suitable period of incubation in order to measure the number of viable intracellular bacteria Previous investigators have usually resorted to differential centrifugation in order to separate extracellular and intracellular bacteria This technique is not entirely quantitative and, as documented in our study, when the ratio of extracellular to intracellular bacteria is high as during the early phase of incubation or when the phagocytosis is reduced, extracellular contamination may markedly obscure the determination of the relatively small numbers of viable intracellular bacteria (2, 7, 14) In addition, bacteria which have become adherent to the external granulocyte wall may not be eliminated by differential centrifugation *Finally, if the leucocyte bacteria* suspension contains erythrocytes which is usual when the granulocytes are prepared from blood samples, immune adherence of bacteria to the erythrocytes may also become a major problem

By our method, extracellular bacteria are effectively controlled by antibiotics which cause no inactivation of intracellular bacteria (13) However, control of extracellular bacteria by antibiotics takes about 10–15 minutes During this period, killing of intracellular bacteria by the granulocytes may significantly obscure the results (13) Accordingly, inhibition of the batericidal activity of the granulocytes is a prerequisite for the determination of the number of viable intracellular bacteria *while extracellular killing* by antibiotics takes place The essence of our method is, therefore, the combined use of phenylbutazone for the inhibition of intracellular *killing of bacteria* and high concentrations of antibiotics for the control of extracellular organisms

The standardization of the bacteria granulocyte and serum concentrations at their optimum and the combined use of phenylbutazone and antibiotics have made available a test system which offers not only a precise evaluation of the phagocytosis but also of the dynamics of the intracellular phase Results of clinical studies show that the test is of particular value in the diagnosis of diseases with various neutrophil granulocyte defects like fatal granulomatous disease of the childhood (12)

In patients with long term septicaemia (12) and virus infections (to be published) the demonstration of reversible defects in the bactericidal activity of the granulocytes is more important, however, suggesting that this method may be of value in the diagnosis of the many clinical circumstances where enhanced susceptibility to infection remains unexplained by current analysis of *host defence* mechanisms

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PATHOGENICITY OF *STAPHYLOCOCCUS AUREUS* MUTANTS IN GENERAL AND LOCAL INFECTIONS

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The pathogenecity for mice of *S aureus*, Cowan I, and derived mutants and revertants was investigated by two methods, one involving intravenous injections and the other, subcutaneous injections in cotton dust Mutants and revertants with reduced production of protein A and α haemolysin, or loss of protein A and coagulase, showed only a slight decrease in pathogenicity compared with the wild type, as determined by the two pathogenicity tests Mutants which lacked protein A, nuclease, coagulase, fibrinolysin, mannitol utilization, and phage type pattern, and mutants producing only nuclease or α haemolysin showed significantly decreased pathogenicity Reproducible results were obtained with both subcutaneous and intravenous injections Similar results were obtained with normal guinea pigs infected intracardially No spontaneous *in vivo* reversion was detected It is concluded that the pathogenicity of *S aureus* depends on the combined effects of many products including protein A

Studies of staphylococcal protein A have indicated that it is a constitutent of the cell wall (4, 7, 30) and, in addition, an extracellular product (4) which reacts directly with the Fc-portion of γG globulins of many mammalian species (8 9 16, 24) Evidence has been presented that protein A may contribute to the pathogenicity of staphylococci Many events to occur in inflammatory reactions to staphylococci might be initiated by the γG-protein A reaction (24) Protein A elicits hypersensitivity reactions of the immediate type in guinea pigs (11) Rabbits develop an Arthus-type reaction after injection of protein A subsequent to administration of human IgG (10) Experiments with Balb/c mice revealed local anaphylactic reactions after injection of 0 1 μg protein A (28) Skin hypersensitivity to isolated protein A was demonstrable in all of the tested adult human subjects Addition of protein A to serum results in a depression of the complement activity (25) Strains of *Staphylococcus aureus* containing large amounts of protein A tend to be more resistant to phagocytosis than strains containing smaller amounts of this protein or none at all (2)

The primary purpose of the present work was to investigate the role of protein A in staphylococcal infections A direct approach to a study of the role of bacterial products is a study of the purified products in biological systems (29) The role of individual characters can also be systematically analysed if characterized mutants deficient in different properties are used In addition to the rela-

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tion of protein A to pathogenicity, the reaction of some other properties—coagulase nuclease, α haemolysin, fibrinolysin, mannitol utilization and phage type pattern—could be investigated

The degree of pathogenicity for guinea pigs was determined as the dose of organisms injected intracardially and capable of killing 50 per cent of the animals (LD50) within a period of 10 days Groups of 10 guinea pigs per dose were used

MATERIALS AND METHODS

Bacteria *S aureus*, Cowan I which produces large amounts of protein A was used throughout the investigation

Mutagenesis *S aureus* Cowan I, was exposed to nitrosoguanidine or ethylmethanesulphonate and screened on nutrient agar plates containing rabbit anti protein A serum for loss of protein A production (6) More than half of all the protein A deficient mutants also lacked nuclease coagulase α haemolysin, fibrinolysin mannitol utilization and the phage type pattern Mutants with various combinations of deficiencies in these factors were also isolated In addition induced revertants of mutants obtained with nitrosoguanidine were isolated after treatment with ethylmethanesulphonate and vice versa.

Media and growth conditions Inocula for animals were obtained by growing the wild type Cowan I or mutants or revertants obtained from it in nutrient broth for about 10 hrs under aeration by shaking to a concentration of 10^9 bacteria/ml For test of *in vitro* growth of different strains nutrient broth and aeration by shaking were used Growth was followed turbidimetrically in a colorimeter and by viable count

Animals Mice of both sexes of the NMRI strain 4 5 weeks old and weighing 15–20 g and guinea pigs (175–200 g) were used

Pathogenicity tests The degree of pathogenicity for mice was measured by

1) Subcutaneous injections of staphylococci in cotton dust performed according to the method described by Noble (1965) except that the cotton plug was inoculated with 10 μl of a broth culture of staphylococci At intervals after inoculation pairs of randomly selected mice were killed and the tissue containing the lesion was excised The excised tissue was homogenized in a tissue grinder with 1 ml of saline and a small quantity of sterile sand Ten fold dilutions of the homogenized suspension were made in saline and viable counts of cocci were done on nutrient agar Zero hr counts were made in all cases by killing two animals immediately after the inoculation of cocci

2) Determination of the dose of organisms injected intravenously and capable of killing 50 per cent of the animals (LD50) within 10 days The 50 per cent end point was calculated according to the method of Litchfield and Wilcoxon (1949) from 4 or 5 doses decreasing by half using groups of 10 mice per dose

RESULTS

In vitro experiments Figure 1 shows the rates of growth of *S aureus* Cowan I, and derived mutants and revertants grown in nutrient broth under aeration by shaking There was no significant difference in growth rate Table 1 gives the properties of the strains shown in Figure 1 *S aureus*, Cowan I, strain 1, a mutant of Cowan I, strain 2, deficient in all properties investigated, and strains 3 and 4 which are revertants of the mutant. All strains included in the *in vivo* experiments described in this paper showed approximately the same growth rate *in vitro*

Subcutaneous infection of mice A typical experiment in which the wild type, the mutant and the revertants shown in Figure 1 and Table 1 were injected subcutaneously in cotton dust is shown in Figure 2 The numbers of viable staphylococci in the lesions at the sites of injection were counted immediately after injection and subsequently at intervals It applies to all the inocula that the number of viable cocci decreased during

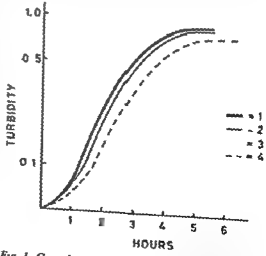

Fig 1 Growth in nutrient broth of Cowan I(1), the mutant (2) and revertants (3 and 4) presented in Table 2

TABLE 1 *Properties of Cowan I (1), a Mutant (2) Derived from Cowan I and Two Revertants (3 and 4) Derived from the Mutant*

Strain	Production of Protein*) A	Nuclease	Coagulase	α haemo lysin§)	Fibrino lysin	Mannitol utilizat	Phage type
1	64	+	+	16	+	+	52/52A/80 81
3	1–2	+	+	2	+	+	52/52A/80/81
4	–	+	—	16	+	+	52/52A/80/81
2	–	—	—	—	—	—	NT †)

*) Reciprocal haemagglutination titre of extracellular *protein A* in a broth culture containing 10^9 colony forming units per ml

§) Reciprocal titre of α hemolysin

†) *NT*, not typable

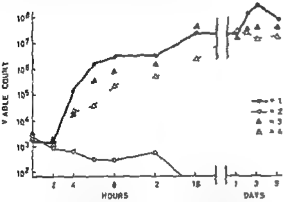

Fig 2 Recovery of staphylococci from the lesions after subcutaneous infection with *cotton dust* of Cowan I (1) the mutant (2) and revertants (3 and 4) presented in Table 2

the first period after inoculation As shown in the figure, the lowest counts for Cowan I and for the mutant deficient in protein A and coagulase were recorded 2 hrs after inoculation After the initial decrease, the numbers of recovered viable staphylococci increased for Cowan I, for the revertant strain 3 with a reduced production of protein A and α haemolysin, and for the revertant strain 4 deficient in protein A and coagulase production There were no significant differences in the rates of increase However, for the mutant, strain 2 which lacked all the pathogenicity properties investigated the viable count was further decreased and no staphylococci were detectable after 15 hrs

TABLE 2 *Bacterial Counts (Colony Forming Units) 24 Hours after Subcutaneous Injection with Cotton Dust of 2–3 10^4 [illegible] aureus Cowan I and Various Derived Mutants and Revertants*

o of rains	Total expts performed	Viable count after 24 hrs (mean value)	Production of Protein A*	Nuclease	Coagulase	α hemolysin§	Fibrino lysin	Mannitol utilization	Phage type
Cowan I)	4	4 5 10^7	64	+	+	16	+	+	52/52A/80 [illegible]
	4	1 4 10^7	1–2	+	+	2	+	+	52/52A/80 8[illegible]
	3	1 3 10^7	—	+	—	16	+	+	52/52A/80 81
	1	$<10^2$	—	+	—	—	—	—	NT†
	1	$<10^2$	—	—	—	16	—	—	NT
	4	$<10^2$	—	—	—		—	—	NT

*) Reciprocal haemagglutination titre of extracellular protein A in a broth culture containing 10^9 colony forming units per ml

§) Reciprocal titre of α hemolysin

†) NT not typable

TABLE 3 *Comparison of LD50 for S aureus, Cowan I and Various Derived Mutants and Revertants*

Strains	LD50 (×10⁸ organisms, mean value)	Production of Protein A*	Nuclease	Coag-ulase	α hemo-lysin§	Fibrino-lysin	Mannitol utiliza-tion	Phage type
n I)	600	64	+	+	16	+	+	52/52A/80/81
	730	1 2	+	+	2	+	+	52/52A/80/81
	1020	—	+	—	[illegible]	+	+	52/52A/80/81
	>3000	—	+	—	—	—	—	N T †
	>3000	—	—	—	16	—	—	N T
	>3000	—	—	—	—	—	—	N T

*) Reciprocal haemagglutination titre of extracellular protein A in a broth culture containing 10^9 colony forming units per ml

§) Reciprocal titer of α hemolysin

†) N T, not typable

Table 2 summarizes the viable counts of a number of mutants and revertants 24 hrs after subcutaneous injections of the staphylococci in cotton dust The highest viable counts were obtained for Cowan I, but mutants in which production of protein A and α-haemolysin was deficient and most of the mutants and revertants lacking both protein A and coagulase gave viable counts of the same order After 24 hrs no bacteria were detectable at the sites of injection of strains producing only nuclease or α-haemolysin or strains lacking all the properties investigated

It applies to all mutants and revertants that bacteria isolated from the subcutaneous lesions were characterized by *in vitro* production of protein A and toxins, and phage type The properties of the isolated bacteria were always the same as those of the injected strain

Intravenous infection of mice Table 3 shows the LD50 results recorded 10 days after intravenous injection into mice of 1 ml of nutrient broth containing staphylococci As shown in the table, mutants with reduced production of protein A and α-haemolysin behaved almost like the wild type Cowan I Strains which lacked both protein A and coagulase showed only a slight decrease in pathogenicity As regards the α haemolysin-positive strain which lacked protein A nuclease, coagulase fibrinolysin mannitol utilization, and phage type pattern and the nuclease-positive strain deficient in all other properties there was a significant decrease in virulence All mice injected with these strains survived a 10 day observation period The same result, survival of all mice, was obtained with the mutants showing a deficiency in all properties investigated

Intracardial infection of guinea pigs The LD50 doses for guinea pigs 10 days after intracardial injection of staphylococci were determined for three strains 1) the wild type, Cowan I, 2) a mutant only deficient in protein A production, and 3) a mutant deficient in protein A, nuclease coagulase fibrinolysin mannitol utilization, and the phage type pattern For the first two strains almost the same LD50 values were recorded approximately 10^9 organisms However in the case of the third strain that was deficient in all properties investigated a value fourty times higher 4×10^{10} was obtained

DISCUSSION

The characters that make *S aureus* a potential pathogen must be many and subtle What we would like to know is the precise role and importance of each known character

Coagulase production has been accepted as the primary criterion for a differentiation be

tween pathogenic strains of staphylococci (*S aureus*) and commensal strains (*S albus* Bergy s Manual 7th ed) In staphylococci correlation between coagulase and protein A production is close Among 700 coagulase positive staphylococcal strains isolated from human subjects 692 produced protein A (5) The capacity of staphylococci to produce protein A may be as closely linked to a mechanism of pathogenesis as is its capacity to coagulate blood plasma Of course, the fact that a substance is produced by all or by the majority of pathogenic strains is no proof of its direct association with virulence however tempting this idea may be A reaction between purified protein A and the Fc part of γG globulin however has been shown to be able to initiate both Arthus reactions and hypersensitivity reactions of the immediate type (24) Protein A has also been shown to have an antiphagocytic effect (2) The original purpose of the present experiments was to further investigate the role of protein A in staphylococcal pathogenicity In addition to the relation of protein A to pathogenicity the relations of some other properties—coagulase nuclease a haemolysin fibrinolysin mannitol utilization and phage type pattern—could be investigated Several reports provide evidence which strongly suggests that staphylococci owe at least part of their pathogenic capacity to some of these factors A direct approach to a study of bacterial products is to study the purified products in biological systems Coagulase (29 19) α haemolysin (1) and other extracellular products of *S aureus* have been shown to cause disease and death when inoculated into experimental animals Many workers have tried to find a connection between the *in vitro* properties of staphylococcal strains and their pathogenicity for man and experimental animals (3 12 13 26 27) The use of mutants in investigations of the role of coagulase α haemolysin and capsular structure in staphylococal pathogenicity has been described (14 15 17) However there is as yet no full and complete explanation of the pathogenic effects of *S aureus* Some of the reports present contradictory evidence regarding the role of different products in *S aureus* pathogenicity

Little direct evidence is available about the virulence of *S aureus* in man (3) The virulence of a microbe is always related to the host species The fact that lesions are produced in one experimental animal does not mean that the organism will be equally virulent in another species However mice and rabbits are the animals most widely used for testing the pathogenicity of *S aureus* Mice were mainly used in this study because of the large number of animals required Since doses of 0 5 mg purified protein A or more given intracardially to guinea pigs cause anaphylactic shock and death (11) some experiments were performed on guinea pigs

Two methods were used in this investigation of the pathogenicity for mice of the staphylococcal strains one in which intravenous injections simulate a generalized infection and the other in which subcutaneous injections simulate a local infection

Numerous attempts have been made with a view to finding a laboratory test that would indicate the pathogenicity of *S aureus* for man In man the number of deaths due to staphylococcal infection is small compared with the frequency of infection (23) The production of subcutaneous lesions in mice after subcutaneous introduction of staphylococci on plugs of cotton dust was intended to simulate the more common type of natural staphylococcal infection in which probably only a few bacteria are initially introduced To produce a lethal generalized infection it was necessary to inject intravenously a very large dose of staphylococci The subcutaneous method using cotton dust has the advantage of requiring a lower infection dose Both methods showed good reproducibility

The experiments described in this paper using characterized mutants and revertants derived from one *S aureus* strain Cowan I permit comparison of the importance of the loss of different pathogenicity factors The effect of a combined loss could also be studied The results obtained are in agree-

ment with those reported in some, but not all, of the papers cited. It is concluded that none of the pathogenicity factors investigated *per se* are responsible for the pathogenic effect of *S aureus* Pathogenicity must be assumed to depend on the combined effects of many products, including protein A

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HAEMOPHILUS IN THE UPPER RESPIRATORY TRACT OF CHILDREN

A Bacteriological, Serological and Clinical Investigation

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The occurrence of *Haemophilus* in nasopharyngeal material from 130 hospitalized children aged 0–7 years has been investigated If a selective medium was used, the carrier rates were very close to 100 per cent The lowest rate was found in children aged less than one year (78 per cent) Out of a total of 137 *Haemophilus* strains isolated, *H influenzae* amounted to 49 per cent, the remaining 51 per cent were shared between the species *H parainfluenzae*, *H haemolyticus* and *H parahaemolyticus* V-factor independent strains were rarely found The methods used for the determination of the growth factor requirements and their importance for the classification of the bacterial strains are discussed An unclarified but constant finding of antibodies to *H influenzae* type *d* antigen in sera from children aged more than nine months is described Such antibodies were not found in children below that age, and were rather infrequent in sera from a group of young adults No correlation was found between the bacteriological findings, the clinical findings and the presence of antibodies to the six *H influenzae* types in sera from the children investigated Thus the investigation does not contribute to the understanding of the clinical significance of any of the *Haemophilus* species

The genus *Haemophilus* has been divided into species (*Pittman* 1931, 1953) using the following criteria the requirements for the growth factors X (haemine) and V (NAD) supplemented with the presence or absence of haemolysis of different blood agar media The species name *H influenzae*, however, has often been used as a general name for all small gram negative rods isolated from throat cultures and forming satellite colonies around a staphylococcus streak on a blood agar plate This is probably due to the fact that the other species of *Haemophilus* are regarded as rare inhabitants of throats From a clinical point of view it has furthermore been stated that it is unimportant to separate the different species Very little is known, however, about the occurrence of the *Haemophilus* species in respiratory tracts The methods which have been used for the determination of the growth factor requirements are numerous, but they often have been inadequate, leaving the question open whether the organisms tested were completely dependent or independent of the growth factors

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Some species of *Haemophilus* may occur in a capsulated form It is generally accepted that such organisms (especially type *b* of *H influenzae*) are pathogenic for man, being the cause of some severe acute infections Although non capsulated bacteria of the genus *Haemophilus* are regarded as a part of the "normal flora" of the upper respiratory tract, numerous investigations have indicated that such strains may be pathogenic in bronchial and sinus disorders (*Mulder et al* 1952 *Allibone et al* 1956, *May* 1965) Whether or not non-capsulated strains of *Haemophilus* may be of any importance in respiratory diseases in younger children is unknown

The present investigation aims at the occurrence of *Haemophilus* species in upper respiratory tracts of children, at the relation to clinical findings and at the presence of antibodies to *H influenzae* capsular antigens in sera of the patients investigated

MATERIALS AND METHODS

The study included all children less than eight years of age that were admitted to the University Clinic of Pediatrics Aarhus kommunehospital in the period from January through March 1971 Clinical examination bacteriological examination of specimens from nasopharynx as well as blood sampling was performed on all children shortly after the admission

Clinical Examination

One clinician performed all the clinical investigations which primarily focused upon ears nose throat oral cavity, and respiratory tract Whenever indicated X rays of the chest and maxillary sinuses were taken Laboratory investigations included estimation of the blood sedimentation rate and haemoglobin as well as total and differential white blood cell counts

A special note was made in the record when antibiotics had been given prior to admission

Bacteriological Examination

1 *Isolation of strains* Nasopharyngeal material taken with a cotton swab on a curved metal rod was plated immediately on two different media 1) on 5 per cent horse blood agar plates subsequently provided with a streak of a staphylococcus culture and 2) on a selective medium consisting of chocolate agar (10 per cent heated bovine blood) containing 300 μg/ml bacitracin as described by *Hovig & Aandahl* (1969) After incubation overnight at 35° C in an atmosphere supplemented with 10 per cent carbon dioxide the plates were examined for growth of *Haemophilus* species and other organisms of possible clinical interest The growth density of *Haemophilus* on the selective medium was recorded as scanty, moderate or heavy

2 *Characterization of strains Haemophilus* suspect colonies were subcultured on 5 per cent horse blood agar plates with a staphylococcus streak for the examination of haemolysis and satellite phenomenon All strains were grown on Levinthal agar plates (*Alexander* 1958) for 18-20 hours at 37° C to observe iridescence due to production of capsular material The *X* and *V* factor requirements were tested in two ways 1) *X* and *V* factor discs (Oxoid) on Brain Heart Infusion Agar (Difco) was used as the standard method Strains independent of the *X* factor by this method were further tested by serial transfers on two defined agar plate media

Medium "XV" TC medium 199 (Difco) 11 g Na oleate 4 8 mg putrescine 3 0 mg β diphosphopyridine nucleotide grade III (Sigma) 3 mg were dissolved in 500 ml of distilled water and 24 mg of haemin (BDH) dissolved in 0 5 ml triethanolamine was added The solution was sterilized through a Millipore filter (0 45 μm) and mixed with equal amounts of a pre autoclaved 2 per cent aqueous agar solution This medium included all nutritional requirements for *Haemophilus* as described by *Buttler* (1962a)

Medium "V" this medium was composed of the same ingredients as Medium "XV" except for the haemine

Six strains were streaked on each agar plate using a reference strain of *H influenzae* as a control All strains were serially cultured and transfered unto both media the "XV" medium served as a growth control The primary inoculum was a single colony from a 24 hours chocolate agar culture The plates were incubated at 37° C in air supplemented with 10 per cent CO_2 and were examined for growth after 24 and 48 hours

3 *Typing of capsulated strains* All strains showing iridescence on Levinthal agar were classified into types according to *Pittman* (1931 1953) by slide agglutination and capsular swelling procedures using the six *H influenzae* typing sera *a f* supplied by Hyland Laboratories of *Los Angeles*

Serological Examination of Sera

Sera from the children were examined for antibodies to all six types of capsulated *H influenzae* The haemagglutination procedure described by *Turk & Green* (1964) was used, with

the modification that the haemagglutination reaction was read from the pattern shown by the sedimented blood cells after incubation for one hour at room temperature The six type strains were provided by Statens Seruminstitut, Copenhagen The antigen sensitized human group O red blood cells were prepared every day and tested for specificity and sensitivity against dilutions of the six typing sera. Two fold serial dilutions of sera from the patients were made in 0 85 per cent saline

A three layer immunofluorescent technique as used by *Brogaard Hansen* (1972) was applied to some sera for determination of the immunoglobulin class of antibodies reacting with *H influenzae* type *d* antigen The antigen was type *d H influenzae* bacteria heat fixed to a slide The first layer was the serum to be tested Monospecific rabbit antisera (1 16) against human IgG, IgA or IgM were used as second layer, and the third layer was a fluorescein-conjugated horse anti rabbit serum (1 70) (Blood Transfusion Laboratories Amsterdam A negative serum was used as control

RESULTS

The study included 130 children of ages between 4 days and 7 years 4 months The distribution of the subjects into three age groups is shown in Table 2

The clinical examinations revealed signs of infection in the respiratory tract of 21 patients In addition to a rise in body temperature the symptoms were in the form of sneezing coughing coating of tonsils swelling of regional lymph nodes respiratory sounds, and cloudy appearance of sinuses on X rays The remaining 109 children had no clinical signs of infections in the respiratory tract Eight children suffered from different systemic diseases In the period prior to admission five had been treated with ampicillin three with sulphonamides and four with G penicillin

Table 1 gives the result of the comparison of the two media used for the isolation of *Haemophilus* The isolation rate was 91 per cent on the selective medium as against 40 per cent on the conventional medium The present study confirms the results of *Hovig* & *Aandahl* (1969) that heavy growth of *Haemophilus* may be present on the selective medium without being observed on the blood agar plates

Table 2 shows the isolation rate of *Haemophilus* in the three age groups The isolation rate from nasopharynx of infants aged less than one year is significantly lower than that in the older groups $(0\,025<P<0\,050)$ Among non carriers two children were treated with ampicillin and two with sulphonamides for a short period prior to admission

The isolation of other possible pathogens is shown in Table 3 *Streptococcus pneumoniae* was the organism most frequently isolated Among non-carriers of *Haemophilus* two patients carried *E coli*, two *Staphylococcus aureus* and one *Klebsiella pneumoniae* No correlation was found between carriage of *Haemophilus* and *Streptococcus pneumoniae*

A total of 137 different strains of *Haemophilus* were isolated In cultures from 19 nasopharyngeal swabs two different strains were found Among the 137 strains, 78 seemed to be independent of the *X* factor (haemine) when tested on Brain Heart Infusion Agar (Difco) using *XV*, *X* and *V* factor discs When retested on the two defined media only 45 of the 78 strains were in fact independent of the *X*-factor Several transfers on the "*V*-medium were in nearly all cases required to eliminate the effect of *X*-factor carried over with the primary inoculum Washing of the cells in saline did not remove this residue of *X* factor In all cases three serial transfers were sufficient to identify whether or not the strains were independent of the *X*-factor Four strains were not stimulated in their growth by these media

Table 4 shows the distribution of the 137 *Haemophilus* strains into five species according to their growth factor requirements on defined media and their ability to lyse horse blood The species *H influenzae* amounts to 49 per cent of the total number of strains The remaining 51 per cent were divided between four species with only four strains being *V* factor independent None of these four strains were haemolytic No further attempt has been made to classify these strains

Determination of the species distribution of strains which could be isolated from the two different primary media showed that no

TABLE 1 *Comparison of Two Media Used for the Isolation of* Haemophilus *in Nasopharyngeal Swabs from Children*

Total No of samples	130		
No of samples with *Haemophilus* as revealed by			
1 Blood agar with a staphylococcus streak	52 (40 %)		
2 Chocolate agar with 300 µg/ml bacitracin	118 (91 %)	scanty growth moderate growth heavy growth	16 % 53 % 31 %

TABLE 2 *Carrier Rates of Haemophilus in Nasopharynx of Children in Different Age Groups*

				Total group
Age group (years)	<1	1–2	>2	0–7
No of subjects	45	28	57	130
Carrier rate of *Haemophilus*	78 %	96 %	98 %	91 %

TABLE 3 *Other Possible Patogens Isolated from Nasopharynx of 130 Children*

Possible pathogens	No of carriers
Streptococcus pneumoniae	31
Haemolytic streptococci*	6
Staphylococcus aureus	5
Escherichia coli	7
Klebsiella pneumoniae	1

* Strains forming soluble haemolysin

TABLE 4 *Distribution in Species of 137* Haemophilus *Strains Isolated from Nasopharynx of Children Aged less than 8 Years*

Total No of strains	137
Haemophilus influenzae	67 (49 %)
Haemophilus parainfluenzae	34 (25 %)
Haemophilus haemolyticus	17 (13 %)
Haemophilus parahaemolyticus	15 (11 %)
V factor independent strains	4 (3 %)

The growth factor requirements were tested on defined media Haemolysis was tested on 5 per cent horse blood agar

specific species was lost if blood agar was used as the only medium

Table 5 shows the percentage of carriers, in the three age groups, harbouring the different species The predominance of the species *H influenzae* increases with age, but in the group of infants aged less than one year, the number of *H influenzae* carriers does not differ significantly from the number of carriers harbouring *H parainfluenzae*

Capsulated strains were always typed, after iridescence had been detected on Levinthal agar If strains forming large mucoid colonies but no distinct irriscence on Levinthal agar plates were tested for agglutination in the typing sera a slow reaction was often noted in one or more of the sera This unspecific reaction reported previously by *Turk & May* (1967) was most frequent in typing serum *f*

Table 6 shows the number of capsulated strains isolated and the distribution of these strains into six sero types Four of the capsulated strains were isolated from infants aged less than one year, one infant being only 4 days old Type *b* was most frequently isolated Two capsulated strains identified as members of the species *H parainfluenzae* according to their growth factor requirements reacted with type *d* and *f* serum, respectively in slide agglutination as well as in capsular swelling tests

The growth density of capsulated *Haemophilus* on the selective medium was in all 11 cases moderate to heavy In spite of this only 4 of these 11 specimens yielded growth of *Haemophilus* when cultured by the routine method

Table 7 shows the occurrence and titres of

TABLE 5 *Occurrence of Different* Haemophilus *Species in the Nasopharynx of 118 Carriers*

Age group (years)	<1	1–2	>2
No of carriers	35	27	56
Haemophilus influenzae	37 % (13)	56 % (15)	70 % (39)
Haemophilus parainfluenzae	34 % (12)	30 % (8)	23 % (13)
Haemophilus haemolyticus	23 % (8)	0	16 % (9)
Haemophilus parahaemolyticus	14 % (5)	15 % (4)	11 % (6)
V factor independent species	3 % (1)	7 % (2)	2 % (1)

The number of carriers is given in brackets
Same individual may be represented in two groups

TABLE 6 *Frequency of Capsulated Strains of* Haemophilus *Isolated from 130 Children Aged less than Eight Years*

Total no of strains	Total no of capsulated strains	No of strains belonging to *H influenzae* type					
		a	b	c	d	e	f
137	11 i e 8 %	1	5	0	0(1)	2	1(1)

The bracketed figures indicate capsulated strains belonging to the species *H parainfluenzae* according to their *X* factor independence but reacting with the respective *H influenzae* typing sera

TABLE 7 *Occurrence and Titres of Serum Antibodies against* Haemophilus influenzae *Type Antigens*

Haemagglutination titre	No of sera with antibodies against type					
	a	*b*	*c*	*d*	*e*	*f*
8	4	9	24	23	22	4
16	1	1	1	18		–
32		1	–	18	–	
64	–		–	12	–	–
128				4		

serum antibodies reacting with *H influenzae* type antigens Sera from 15 patients were not tested All titres measured except those for antibodies reacting with type *d* antigen were very low Only one infant had a serum titre higher than 16 to any of the other antigens viz a six days old infant having a titre of 32 to type [illegible] antigen

Antibodies reacting with type *d* antigen occurred surprisingly common and the titres were fairly high The mean titres for antibodies reacting with type *d* antigen are given in Tables 8 and 9 As seen from these tables, there is a sudden rise in the mean titre about the age of *9* to *10* months Only one infant aged less than 9 months was positive as regards this type of antibody (titre 2), while in the group of children aged more than 9

TABLE 8 *Mean Titres for Antibodies Reacting with Type* d *Sensitized Red Blood Cells in Different Age Groups*

Age group (years)	<1	1 2	>2	20–29
No of subjects	42	26	47	43
Mean titre	4	29	27	7

TABLE 9 *Mean Titres for Antibodies Reacting with Type* d *Sensitized Red Blood Cells in a Group of Children Aged less than One Year*

Age group	0–9 months	9 12 months
No of subjects	32	10
Mean titre	0*	18

* Only one child had a positive reaction (titre 2)

months and less than 7 years only four were negative as regards this specific antibody These groups of children were compared with a group of young adults consisting of 43 persons aged 20 to 29 years The results of the estimation of titres of antibodies to type *d* antigen for this group are also given in Table 8 Occurrence of antibodies to type *d* antigen in sera from these persons were not so frequent and the titres were generally low as compared to the group of children aged more than nine months The three layer immunofluorescent technique applied to ten positive sera from children revealed that the active antibody belongs to the IgM globulin class

In two children only, harbouring capsulated strains in the nasopharynx homologous antibodies were found in the sera, they showed low titres

A comparison of the clinical and bacteriological findings showed that the carrier rates of *Haemophilus* were equal in the groups with and without clinical signs of infection in the respiratory tract A capsulated *Haemophilus* was found in a single child with signs of infection in the respiratory tract but no homologous antibodies were demonstrated in the serum at the time of the bacteriological sampling No particular species of *Haemophilus* was found in nasopharynx of patients with clinical signs of infection

DISCUSSION

The results of the present study support the generally accepted fact that bacteria of the genus *Haemophilus* belong to the 'normal flora' of the upper respiratory tract of children Taking only one sample per subject the isolation rate using a selective medium was nearly 100 per cent (Tables 1 and 2) Although the lowest isolation rate was found in a group of infants aged less than one year (78 per cent) *Haemophilus* were isolated from nasopharynx of newborn babies 4 5 and 6 days after birth

Using the same media the present study showed rates of isolation of *Haemophilus* somewhat higher than those observed by *Hovig & Aandahl* (1969) With blood agar these authors found an isolation rate of 5 per cent as compared to the present 40 per cent and with the selective medium they found a rate of 61 per cent as compared to the present 91 per cent Both studies comprised patients hospitalized for different diseases The present study however was restricted to individuals less than eight years of age

Bacteria belonging to the species *H influenzae* were most frequently isolated amounting to 49 per cent of the total number of strains The present study shows however that the species *H parainfluenzae*, *H haemolyticus* and *H parahaemolyticus* occur in considerable numbers too (Table 4) In fact in the group of infants aged less than one year the frequency of *H influenzae* and *H parainfluenzae* was almost identical V factor independent strains were rarely isolated These results are in contrast to results of investigations carried out by other authors It has been emphazised that bacteria belonging to the species *H parainfluenzae* and *H haemolyticus* are rare findings (*Masters* 1958 *Turk* 1962 *De Leeuw Frans et al* 1964 *Branson* 1968) In these investigations a complex medium was used as a basic medium for the determination of the growth factor requirements A comparison of the two methods applied in the present study shows, however that a complex medium is unsuited for this purpose Only 58 per cent of the strains apparently X factor independent when tested on a complex medium, were in fact independent when retested on a well defined medium Furthermore the latter method revealed a clear cut difference between X factor-dependent and X factor independent strains

Out of a total of 137, four strains were not stimulated in their growth by the medium designated XV These four strains need further growth factors in addition to the nutritional requirements reported by *Butler* (1962a) but they have not been further investigated in the present study

The strains isolated in this investigation have been divided according to their growth

factor requirements and their ability to lyse horse blood. Recently, the requirement for incubation in an atmosphere with a raised CO_2 content has been used in the establishment of one and proposal of two additional species under the genus *Haemophilus*: *H. aphrophilus* (*Khairat* 1940, *Boyce et al.* 1968), *H. paraphrophilus* (*Zinnemann* 1968), and *H. paraphrohaemolyticus* (*Zinnemann et al.* 1971). On the other hand, the identification criteria of these species are probably still insufficient. The biological background for the increased CO_2 requirement is unknown and the importance of CO_2 as a growth factor has to be further investigated. All strains in this study were isolated from agar plates incubated in a 10 per cent CO_2 atmosphere and some of them might therefore belong to the species mentioned.

The carrier rate of capsulated strains (Table 6) is identical to that previously found by other authors. The total number of capsulated strains is not high, but as previously reported from Denmark by *Engbæk* (1949), *H. influenzae* type *b* is the most frequent type.

Among eleven capsulated strains, two belonging to the species *H. parainfluenzae*, were found to react with *H. influenzae* typing sera *d* and *f* respectively. Capsulated strains of *H. parainfluenzae* is reported to be rare findings (*Turk & May* 1967), and an antigenic relationship to the capsular antigens of *H. influenzae* has not been reported previously. Whether these two strains could be genetically related to capsulated *H. influenzae* is not known. A close relationship was suggested by the findings of *Butler* (1962b) who isolated an *X* factor independent mutant indistinguisable from *H. parainfluenzae*, from a strain of *H. influenzae*.

The growth density of capsulated strains was always recorded as moderate to heavy on the selective medium but out of 11 isolates only four gave visible growth on the blood agar plate. The finding of *Haemophilus* on this medium is probably dependent upon the number and nature of the other microbial organisms composing the flora of the upper respiratory tract and not only upon the relative number of *Haemophilus*. The need for a selective medium is obvious when *Haemophilus* is searched for as a possible pathogen.

Antibodies that might be related to infection caused by capsulated *Haemophilus* were rarely demonstrated in this investigation, and it is not surprising. Thus, *Wood et al.* (1963) have shown that, during an inflammation caused by *Haemophilus*, titres of antibodies to capsular antigens rise early and fall again within a few weeks.

The constant finding of antibodies reacting with type *d* antigen in serum from children aged more than nine months is unexpected. Antibodies to type *d* in many sera were also demonstrated by *Turk & Green* (1964) who used the same technique. At present an explanation to these findings is not apparent. It is rather inconceivable that these antibodies are due to an infection with capsulated *H. influenzae* type *d*, in view of the fact that this type has been found to be rare in this study as well as in several surveys (*Engbæk* 1949, *Dawson & Zinnemann* 1952, *Masters et al.* 1958).

The present study does not contribute to the understanding of the clinical significance of the *Haemophilus* species. The importance of capsular *H. influenzae*, especially type *b*, in producing meningitis and severe acute respiratory tract infections as epiglottitis is well known. *Pittman* (1953) and *De Leeuw Frans et al.* (1964) have suggested a clinical significance of the species *H. parahaemolyticus* as being a possible cause of some cases of pharyngitis and stomatitis. In the present study, the carrier rates of *Haemophilus* could not be correlated to clinical signs of infection, and no particular species was found in the nasopharynx of patients with infection. Capsular strains were found in patients with systemic diseases and in a child only four days after birth. Their presence did not cause any detectable signs of infection. This raises the question whether or not there are any specific virulence factors, in addition to

capsule production, associated with strains causing infections A further investigation of strains of obvious pathogenicity might be helpful in the elucidation of this problem

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STRUCTURAL ROLE OF RNA IN SEMLIKI FOREST VIRUS NUCLEOCAPSID

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Semliki Forest virus nucleocapsids were isolated from purified virus. The nucleocapsids were variously treated with pancreatic ribonuclease and the effect of the treatment studied by sucrose gradient analysis and electron microscopy. After digestion of about 20 per cent of the nucleocapsid RNA the size of the particle had decreased from the original 39 nm to 32 nm. Heavy RNase treatment causing the loss of 50 per cent or more of the RNA did not affect the size much further. When examined by negative staining the small size nucleocapsid showed a layered structure with a central, stain resistant core of about 16 nm and an outer capsid like layer, 3 to 5 nm thick. Annular structures, 7 to 8 nm in diameter, were often discernible, but the structural symmetry of the capsid could not be established.

The group A arboviruses are medium-sized, enveloped RNA viruses. The structure of the near spherical nucleocapsid has not yet been established but cubical symmetry has been suggested by most investigators. Based on electron microscopic evidence, a 32 or 42-capsomere icosahedral symmetry for the nucleocapsid of Semliki Forest virus (*Osterrieth* 1968) and a 32 capsomere symmetry for that of Sindbis virus (*Horzinek and Mussgay* 1969) have been proposed.

On the other hand the nucleocapsid of group A arboviruses shows features different from those of known cubical viruses. One of these is the heterogeneity in the nucleocapsid size, the diameter values being reported vary between 30 and 45 nm (e.g. *Klimenko* 1965, *Friedman and Berezesky* 1967, *Osterrieth* 1968, *Simpson and Hauser* 1968a, b, *Horzinek and Mussgay* 1969, *Kääriäinen, Simons and v. Bonsdorff* 1969, *Acheson and Tamm* 1970a). Another feature is the accessibility of the nucleocapsid RNA to pancreatic ribonuclease (*Burge and Pfefferkorn* 1968, *Sreevalsan and Allen* 1968, *Strauss et al.* 1968, *Dobos and Faulkner* 1969, *Friedman and Grimley* 1969, *Tan, Sambrook and Bellett* 1969, *Acheson and Tamm* 1970b, *Kääriäinen and Söderlund* 1971).

The present study shows that digestion of part of the Semliki Forest virus nucleocapsid RNA leads to the formation of a considerably smaller particle. The small-size nucleocapsid shows structural details not discernible in the larger untreated one.

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METHODS

Virus. The cultivation and purification of radioactively labelled or unlabelled Semliki Forest virus (SFV), prototype strain, has been described earlier (*Kääriäinen et al.* 1969).

Isolation of nucleocapsids. The purified labelled virus (2 to 4 mg of protein) was mixed with an equal volume of 2 per cent aqueous Nonidet (Shell Chemical Co.). After 30 minutes incubation the mixture was centrifuged in 15–30 per cent w/w

sucrose gradient in 0 1 m NaCl 0 05 M Tris, pH 7 4 (TN) for 3 hours at 25,000 rpm in Spinco SW 27 rotor The nucleocapsid fraction was identified by radioactivity and electron microscopy as described earlier (*Kaariainen et al* 1969)

Ribonuclease treatment of nucleocapsids RNase treatment was performed according to *Kaariainen and Soderlund* (1971) Nucleocapsids in sucrose were mixed with varying amounts of pancreatic ribonuclease (EC 2 7 7 16 Worthington Biochemical Corp, Freehold, N J) and incubated at 20° C for the desired times RNase treatment on electron microscopic grids was performed by applying a drop of the nucleocapsid fraction on the grid After removal af the drop with filter paper a drop of RNase, at the desired concentration, was put on the grid and allowed to act for various periods of time The RNase was then removed and the sample negatively stained

Electron microscopy For negative staining, a drop of the specimen was placed on a carbon coated grid and allowed to settle The drop was removed with fitler paper and immediately replaced with the negative stain (potassium phosphotungstate (KPT), pH 7 0) After staining for 45 seconds the excess stain was removed with filter paper For positive staining a 1 per cent aqueous solution of uranyl acetate was allowed to act for 5 minutes on the grid and the excess stain was washed off with distilled water or TN buffer

Electron micrographs were taken with a Siemens Elmiskop I A electron microscope at original magnifications of 20,000 to 60,000 ×, calibrated by use of a carbon grating 54 863 lines/inch (Ladd Research Industries, Inc, Burlington, Vermont)

Particle measurements were made on areas of electron micrographs (120,000 to 200 000 ×) which showed the best correspondence of stain distribution All particles within such areas were measured by means of a measuring lens to an accuracy of 0 2 mm Two perpendicular measures were determined for each particle (the maximum and minimum diameters) and the geometric mean of these two values was taken as the diameter of the particle The mean diameter and standard deviation were determined in each sample and the significance of the observed size differences was analysed using the Student test

Densitometric tracing Electron micrographs of both untreated and RNase treated nucleocapsids enlarged 400 000 times were analysed in a Chromoscan MK II densitometer (Joyce, Lobel and Co, Ltd Team Valley England) at visible reflectance The tracing was performed using a 3×1 mm aperture and the result recorded with a gear ratio of 1 to 3 or 1 to 9

RESULTS

The untreated nucleocapsid Semliki Forest virus nucleocapsids, isolated from purified virus were spherical to slightly oval when negatively stained The variation in particle diameter within each nucleocapsid batch was in the range of ± 4 nm of the mean diameter value The mean nucleocapsid diameter of different batches varied between 38 and 40 nm, and the mean diameter of the pooled material (5 batches, 313 particles) was 38 9 nm

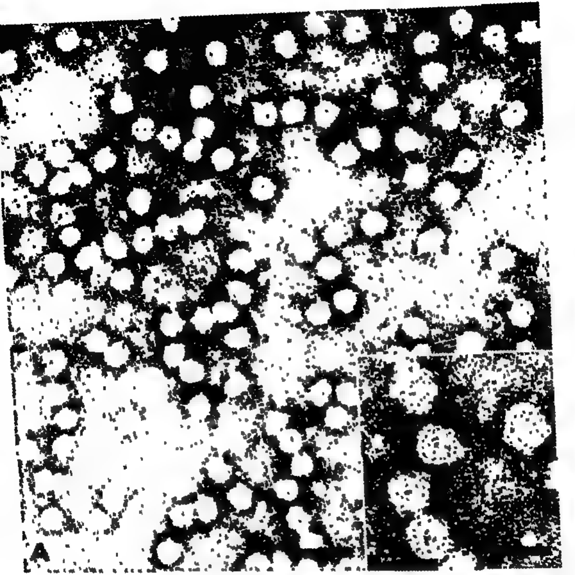
The surface of the negatively stained nucleocapsids did not reveal any distinct details (Fig 1 A) No structures suggesting the presence of morphological subunits could be found At higher magnification (Fig 1 B) the surface of the particles showed a rather finely granular mesh No stain penetration into the nucleocapsids was discernible and "empty" particles were completely absent

TABLE 1 *Effect of RNase on the Size of SFV Nucleocapsid*

RNase treatment	Number of particles measured	Mean diameter[a] and standard deviation in nm
Exp A		
Untreated	81	38 8±2 [illegible]
20 μg/ml 20 min	142	33 1±2 1
20 μg/ml 40 min	92	32 6±1 [illegible]
20 μg/ml 60 min	53	31 8±1 6
20 μg/ml 20 min resedimented	48	30 8±2 2
Untreated resedimented	33	38 4±1 8
Exp B		
Untreated	73	38 1±1 4
1 μg/ml 1 min resedimented	57	32 1±1 4

a) The particle diameters were determined as described in Methods

RNase treatment of the nucleocapsid Radioactively labelled nucleocapsids were treated with RNase at 20 μg/ml for 20 minutes and subsequently analysed by sucrose gradient centrifugation (Fig 2) The nucleo-



... ...ation after Nonidet P40 ... 40 nm in diameter Magnifications

capsids were recovered in one distinct band at 100 S whereas the untreated control nucleocapsids sedimented at 150 S The RNA content of the nucleocapsid, as determined by *Kaariainen et al* (1969) is 38 per cent Using this value, the amount of RNA digested could be calculated from the altered radioactivity ratio of protein to RNA The treated nucleocapsids were found to contain about 19 per cent or half of the original RNA

Specimens for electron microscopy were taken from the peak fractions containing the RNase treated nucleocapsids and stained negatively with KPT, pH 7 0 They were found to contain near-spherical particles which were considerably smaller than the corresponding untreated ones (Table 1 lines

sucrose gradient in 0.1 m NaCl, 0.05 M Tris pH 7.4 (*TN*) for 3 hours at 25,000 rpm in Spinco SW 27 rotor. The nucleocapsid fraction was identified by radioactivity and electron microscopy as described earlier (*Kaariainen et al.* 1969).

Ribonuclease treatment of nucleocapsids. RNase treatment was performed according to *Kaariainen and Söderlund* (*1971*). Nucleocapsids in sucrose were mixed with varying amounts of pancreatic ribonuclease (EC 2.7.7.16 Worthington Biochemical Corp., Freehold, N. J.) and incubated at 20° C for the desired times. RNase treatment on electron microscopic grids was performed by applying a drop of the nucleocapsid fraction to the grid. After removal af the drop with filter paper a drop of RNase, at the desired concentration, was put on the grid and allowed to act for various periods of time. The RNase was then removed and the sample negatively stained.

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The surface of the negatively stained nucleocapsids did not reveal any distinct details (Fig. 1 A). No structures suggesting the presence of morphological subunits could be found. At higher magnification (Fig. 1 B) the surface of the particles showed a rather finely granular mesh. No stain penetration into the nucleocapsids was discernible and "empty" particles were completely absent.

TABLE 1. *Effect of RNase on the Size of SFV Nucleocapsid*

RNase treatment	Number of particles measured	Mean diameter[a]) and standard deviation in nm
Exp. A		
Untreated	81	38.8 ± 2.2
20 µg/ml 20 min	142	33.1 ± 2.1
20 µg/ml 40 min	92	32.6 ± 1.2
20 µg/ml 60 min	53	31.8 ± 1.6
20 µg/ml 20 min resedimented	48	30.8 ± [illegible].2
Untreated, resedimented	33	38.4 ± 1.8
Exp. B		
Untreated	73	38.1 ± 1.4
1 µg/ml 1 min resedimented	57	32.1 ± 1.4

a) The particle diameters were determined as described in Methods.

RNase treatment of the nucleocapsid. Radioactively labelled nucleocapsids were treated with RNase at 20 µg/ml for 20 minutes and subsequently analysed by sucrose gradient centrifugation (*Fig. 2*). The nucleo-

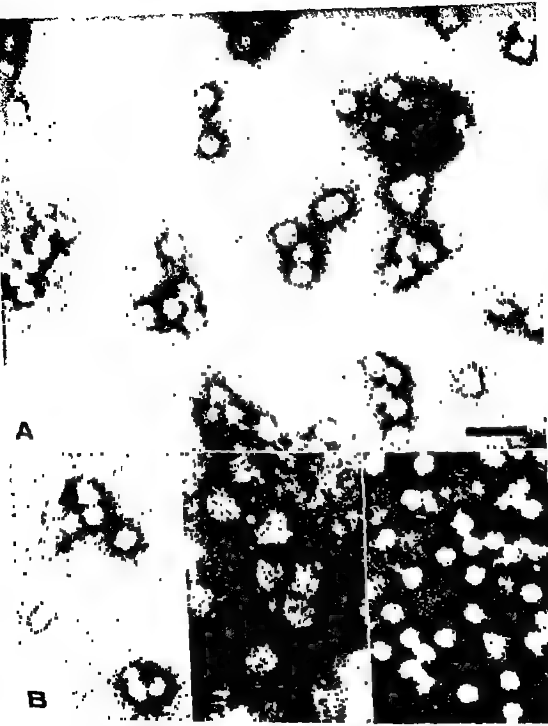

Fig 3 Ribonuclease treated SFV nucleocapsids stained negatively with KPT (pH 7.0) They often reveal

D) Exceptionally large irregular particles

(C) RNase treatment A 20 μg/ml, 40

30 per cent sucrose gradient C 1 μg/ml,

grid All micrographs 150,000 ×, bar

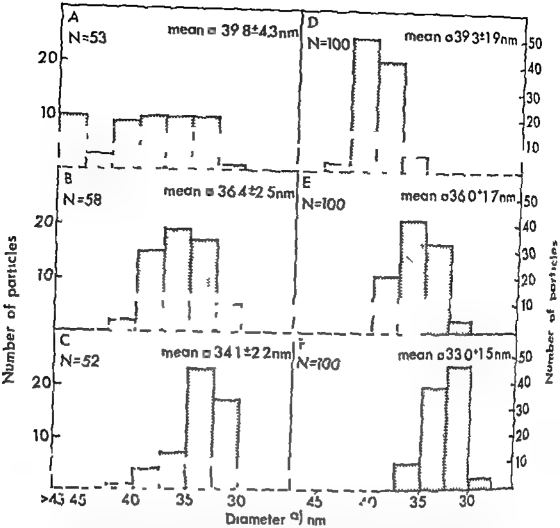

Fig 4 Histograms demonstrating the effect of ribonuclease treatment on the SFV nucleocapsid size. Panels A to C show the size distribution of nucleocapsids treated with 1 µg/ml of RNase for 10 seconds 1 min and 5 min, respectively. Panel D represents untreated nucleocapsids and panels E F respective nucleocapsids treated with 10 µg/ml (E) and 100 µg/ml (F) of RNase for 10 min on electron microscopic grids
N — number of particles measured
a) the diameters of the particles were determined as described in Methods

20 per cent of the nucleocapsid RNA had been digested. Electron microscopy revealed that these nucleocapsids were small having a mean diameter of 32 nm which is close to that obtained after heavy RNase treatment (Table 1, line 8). It would seem that the decrease in size occurs in connection with digestion of the best accesible RNA and that further loss of RNA does not affect the size materially.

If samples for electron microscopy were taken immediately after addition of the RNase (1 µg/ml) they were found to contain particles with a mean diameter similar to that of untreated particles (Fig 4 panel A). The size distribution of the particles in this material was however, quite different due to the occurrence of exceptionally larger particles (Fig 3C). After one minute such large particles were no longer found and a small decrease in the mean diameter had occurred (Fig 4 panel B). The distorted ap-

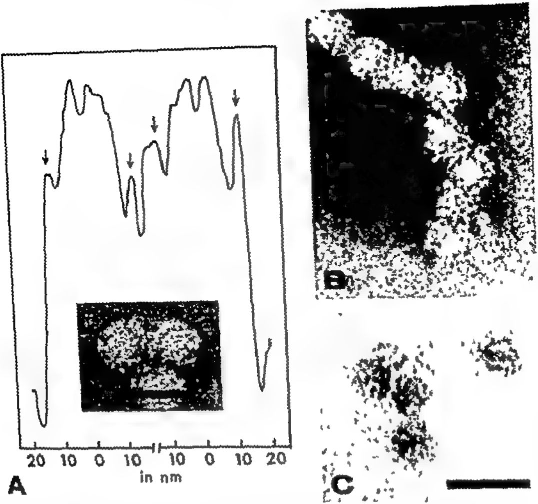

Fig 5 A Densitometric tracing of two adjacent RNase treated SFV nucleocapsids (inset) The arrows point to the peaks which indicate the presence of an outer layer in the particles Treatment and staining as in Fig 3 A SFV nucleocapsids (B and C) treated as described in Fig 3 B The particles have been dialysed against distilled water for one hour before staining negatively with KPT (B) and positively with uranyl acetate (C) Magnification 400,000 × for all micrographs, bars 50 nm

pearance as well as the rapid disappearing of the very large particles suggest that they represent disintegrating nucleocapsids At 5 minutes the mean diameter had decreased considerably (Fig 4, panel C)

The distributions of the diameters as seen in Fig 4 panels A to C are somewhat skew, but they do not indicate that the samples were composed of mixtures of small and large particles Similar results were obtained when the RNase treatment was performed on electron microscopic grids (Fig 4, panels D to F and Fig 3 D) Under these conditions the size decrease of the nucleocapsids occurred more slowly and so higher RNase concentrations were used

Morphology of the RNase treated nucleocapsid The small-size nucleocapsid obtained with RNase treatment showed, when negatively stained, morphological features different from those of the untreated one The difference seemed mainly to be caused by a partial stain penetration into the small nucleocapsid The RNase treated particles frequently revealed a central core, about 16 nm in diameter The stain-rich zone sur-

rounding the core was 3 to 5 nm thick The outermost layer, also 3 to 5 nm thick, seemed to be more stain resistant Densitometric tracing of the particles also clearly demonstrated their layered structure (Fig 5 A) Positive staining with uranyl acetate also enabled an outer layer to be visualized (Fig 5 C) In this case, the layer was very weakly stained in contrast to the heavily stained centre with a diameter of about 25 nm

The structure of the outer, capsid like layer could not be resolved Some circular structures, 7 to 8 nm in diameter, with stain spot centres were discernible in both negatively (Fig 5 B) and positively (Fig 5 C) stained particles However, the number of 'subunits' found on one particle was never sufficient to indicate their possible arrangement

DISCUSSION

The Semliki Forest virus nucleocapsid contains 38 per cent RNA and 62 per cent protein (*Kääriäinen et al 1969*) The molecular weight of the RNA is close to 4×10^6 daltons (*Cartwright and Burke* 1970, *Friedman* 1971) Apparently, only one type of polypeptide (mw 34,000) is present in the nucleocapsid (*Simons and Kääriäinen* 1970 *Acheson and Tamm* 1970c)

Recent results obtained with X ray diffraction indicate that the nucleocapsid of Sindbis virus within the virion is close to 41 nm in diameter (*Harrison et al* 1971) In this study the mean diameter of the nucleocapsid isolated from purified virus was 38 9 nm Although the conditions of the virus purification nucleocapsid isolation and negative staining were kept constant different mean diameters were obtained from each nucleocapsid batch These results suggest flexibility in the nucleocapsid construction This is also indicated by the observation that slightly acid pH (6 2) causes the SFV nucleocapsid to decrease to 32 nm in diameter without loss of protein or breakage of the RNA (*Söderlund et al* 1972)

The decrease in size of the nucleocapsid caused by RNase treatment is of the same order as that caused by acid pH A possible explanation of the formation of the small particle would be that a whole outer protein layer is removed during the RNase treatment This seems, however, highly improbable if the observed gradual decrease in size of the particle during mild RNase treatment or RNase treatment on the electron microscope grid is taken into account Previous biochemical analysis of the RNase treated SFV nucleocapsids has shown that at most 25 per cent of the protein could be lost (*Kääriäinen and Söderlund 1971*) The decrease in size of the nucleocapsid cannot therefore be explained by protein loss but it seems rather probable that digestion of part of the RNA causes a rearrangement of the nucleocapsid protein subunits which leads to the formation of the small particle That the pH induced reduction in size occurs without loss of protein shows that there is space enough for such a change to occur (*Söderlund et al* 1972)

In the small particle, a 16 nm core corresponding to that of Sindbis virus nucleocapsid (*Horzinek and Mussgay* 1969) can be demonstrated The chemical nature of the core is not known Using the partial specific volume of RNA 0 55 g/cm³ (*Markham* 1962), it can be calculated that the residual RNA alone could form the core The presence of protein in the core cannot of course be excluded

The outer 3 to 5 nm thick layer is apparently mostly protein This is suggested by its weak staining with uranyl acetate in contrast to the central area Uranyl acetate is known to bind to RNA better than to protein (*Valentine* 1962) The location of protein on the surface of the nucleocapsid is further indicated by the ability of pronase to digest most of the protein with ease (*Kääriäinen and Söderlund* unpublished data)

The change in the structure of the nucleocapsid caused by RNase digestion suggests that RNA forms part of the capsid and that it somehow stabilizes the protein framework That protein RNA interactions are necessary for its structural integrity is supported mainly

by the following observations (i) the nucleocapsid exists in its large form only if the RNA is not degraded (ii) No empty capsids have been found in the virus preparations at different stages of purification or in thin sections of infected cells (e g *Acheson and Tamm* 1967 and own unpublished data) Whether protein subunit interactions play a role for the nucleocapsid stability remains unestablished It is possible that at least the small nucleocapsid gains its stability through such interactions The location of RNA in the capsid layer of the particle is unknown The constant failure to demonstrate any surface lattice in the large particle by negative staining could be explained by assuming that part of the RNA extends up to the surface of the particle and thus masks the visibility of the subunits

The association of nucleic acid with the capsid protein has been studied especially in some isometric plant viruses It seems that in at least some of these viruses RNA is present within the capsid extending close to the particle surface and that it also is directly bonded to the protein supporting the capsid structure (for references see *Kaper* 1971) In cucumber mosaic virus (CMV) the inter protein subunit linkages seem to be very weak or completely absent and the structural integrity to depend exclusively on protein RNA interactions (*Kaper and Geelen* 1971) The RNA of CMV can be degraded *in situ* by ribonuclease (*Francki* 1968 *Kaper and Geelen* 1971) leading to the disintegration of the particle (*Francki* 1968)

The present results do not give any explanation about the nucleocapsid structure which would clarify the mechanism of the decrease in size The general principles of the construction of cubical viruses (*Caspar* 1965) seem difficult to apply to this case It can however be stated that this nucleocapsid representing the proposed togavirus group possesses characteristics not common to the hitherto known cubical viruses

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HAEMATOLOGICAL CHANGES AFTER INJECTION OF ENDOTOXIN INTO WARFARIN-TREATED RABBITS

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A comparison was made of haematological changes in warfarin sodium treated and in untreated rabbits, given daily intravenous injections of endotoxin from *Salmonella typhi* for 15 days Two groups received a low endotoxin dose and two groups a higher dose Total and differential counts made prior to and 1, 4, and 24 hrs after the 2nd, 8th, and 15th endotoxin injection showed that the considerable changes of the leukocyte count were dominated by fluctuations of the polymorphonuclear cells Low endotoxin doses resulted in only a slight change in the mononuclear counts The higher dose resulted in marked changes which did not parallel those of the polymorphonuclear cells Following the low endotoxin dose no difference was found between warfarin sodium treated and untreated rabbits Following the higher dose a difference was indicated with regard to the polymorphonuclear cells, especially for the response recorded 1 hr after each injection A decrease was found in the volume of packed red cells, which was recorded every other day in the experimental period The fall was most marked in the warfarin sodium treated rabbits, the difference being significant for those receiving the higher dose The erythrocyte sedimentation rate which was recorded every other day, increased in all rabbits, the increase being significantly higher in the warfarin sodium treated than in the untreated rabbits—irrespective of the size of the endotoxin dose

Intravenous injection of endotoxin provokes an increased intravascular coagulation (15) Each injection is followed by changes in the number of circulating leukocytes and platelets Repeated injections also give haemolysis and haemolytic anemia, which are probably secondary to intravascular coagulation (2)

The trigger mechanism of the endotoxin induced increased intravascular coagulation is still uncertain (9) In the rabbit the platelets appear to serve as a mediator of endotoxin (9 29) but in some experiments the coagulation also seems to be triggered in the presence of thrombocytopenia (8, 9) The leukocytes, especially the polymorphonuclear cells, may also be involved in the trigger mechanism, but their role in this mechanism is still very controversial (9, 26) Furthermore, the polymorphonuclear cells seem to phagocytize fibrin and/or fibrinogen breakdown substances formed during the coagulation (25), the endogenous pyrogen and other factors from these cells being released after endotoxin injections (13, 20)

Pretreatment with heparin or warfarin sodium prevents the increased coagulation (4, 12), heparin also prevents a fall in packed cell volume and a rise in plasma haemo-

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globin (1) The anticoagulants, however, do not prevent a drop in the number of platelets after endotoxin or Liquoid injection (6, 22, 28), nor do they influence the endotoxin-induced leukocyte pyrogen response (12, 15) The reports concerning the influence on the endotoxin-provoked changes in the leukocyte count are contradictory (10, 12)

In the present paper a record is made of the circulating leukocyte count following injection of endotoxin into tolerant and non-tolerant rabbits pretreated with warfarin sodium Moreover, the volume of packed cells and the sedimentation rate are examined

MATERIALS AND METHODS

Animals Four previously described groups (15) of normal rabbits (I A, I, II A and II), 6 animals in each, were used One rabbit from groups I A, I, and II died during the experimental period (15)

Endotoxin Bacto Lipopolysaccharide W (L P) from *S typhi 0901* (Difco) (control No 473454) was stored, dissolved, and intravenously injected as described (15)

Anticoagulant treatment with warfarin sodium (warfarin), and Thrombotest determination of the coagulation activity in per cent (TT%), were carried out as described in (14) The rabbits of groups I A and II A were randomly chosen for anticoagulant treatment

Blood samples were obtained from the lateral ear vein

Volume of packed red cells (V P C) and erythrocyte sedimentation rate (E S R) were determined by the technique of *Wintrobe* (35) prior to treatment and every other day of the immunization period

Total leukocyte count was performed by the usual technique in 25 mm³ venous blood diluted with 475 mm³ Türk's dilution fluid A Bürker counting chamber was used and 32 III squares (1/5 mm³ volume) were counted Records were obtained from all rabbits prior to endotoxin immunization, and immediately prior to and 1, 4 and 24 hrs after, the 2nd, 8th and 15th L P injection, respectively The preparations were made and examined by a trained technician who did not know to which rabbit the specimen belonged The total leukocyte count (per mm³) of each rabbit prior to immunization was stated as 100 per cent, and the subsequent leukocyte counts in per cent of the preimmune count

Differential leukocyte count was made from film stained with May Grunwald Giemsa (Pappenheim) stain The counts were performed by a blind technique from all the blood samples in which total leukocyte counts were made, and determined from 200 consecutive cells enumerated on each film. According to *Sjovall* (33), the pseudoeosinophile eosinophile and basophile cells were grouped in one group i e *the polymorphonuclear cells*, and the lymphocytes and monocytes in another group i e *the mononuclear cells* The distribution of leukocytes between the two groups was calculated as a percentage For each blood sample the absolute count per mm³ of polymorphonuclear and of mononuclear cells was calculated according to the corresponding leukocyte count of the sample The preimmune count of mononuclear and polymorphonuclear cells, respectively, of each rabbit was stated as 100 per cent, and subsequent counts in per cent of the preimmune count

Antibody determination in serum from each of the rabbits was carried out as previously described (16)

Pyrogenic response to the L P injections was recorded in each rabbit as described by *Haug* (15)

Statistics The value of a group is taken as the arithmetical mean of the individual values One randomly chosen rabbit from group II A, and one from groups I A I, and II which died during the experimental period, were excluded from the statistical analysis Analysis of variance is performed by the method of *Scheffé* (30) A 5 per cent (0 05) level of significance was used throughout.

EXPERIMENTS AND RESULTS

All rabbits were given a daily intravenous injection of L P at the same hour each morning for a period of 15 days The rabbits of groups I A and I were randomly chosen for a daily dosage of 0 2 mcg L P/kg body weight (b w) and those of groups II A and II of 1 0 mcg/kg b w The recipients of equal doses were experimented on in succession taking two by two One rabbit was randomly chosen from each of the two different groups

None of the rabbits, except one from group I A (No 229) and one from group I (No 232), had a preimmune antibody titre of 2⁴ or higher which was regarded as indicating the presence of a specific antibody to L P

Three days prior to immunization with L P and during the immunization period the rabbits of groups I A and II A were treated with warfarin Prior to warfarin treat

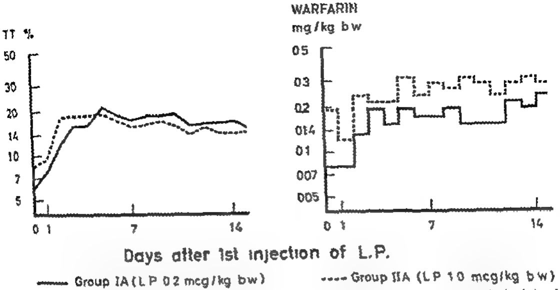

Fig 1 The coagulation activity (TT%) (left) and the warfarin dosage (mg/kg b w) (right) of groups I A and II A receiving daily intravenous injections of endotoxin (L P), 0 2 mcg/kg b w and 1 0 mcg/kg b w respectively

ment the TT% of the individual rabbits including those of groups I and II, was about 100 The daily TT% levels and warfarin dosage of the two warfarin treated groups I A and II A, are shown in Fig 1 For the whole experimental period the mean TT% of groups I A and II A was 14 9 and 15 5, respectively and the mean warfarin dosage 0 17 and 0 24 mg/kg b w, respectively Four of all individual TT% values determined on alternate days were above 30 (range 31–38) The TT% values are not corrected for V P C changes during the experimental period

The V P C Values

The preimmune mean V P C value of the rabbits in groups I A and I was 41 2 (range 36–45) and 41 6 (range 37–48), respectively and in groups II A and II 43 2 (range 41–48) and 41 0 (range 37–42) respectively There was no significant difference between the values of any of the groups During the immunization period the V P C values of all groups decreased (Fig 2) especially those of the warfarin treated groups It should be noted that the decrease in the latter groups is most pronounced in the first few days of immunization. Approximately the same results are obtained when median values of each group are used A comparison by analysis of variance between the individual values of groups I A and I, and between those of groups II A and II, showed no significant main effect of warfarin treatment A significant combined effect (interaction) of the warfarin treatment and the number of L P injections was found on comparison of the values of groups II A and II, but not of groups I A and I

The E S R Values

Prior to L P injections the individual E S R of all rabbits was within the range 0–2 mm/hr During the immunization period there is an increase in the mean E S R values of all groups, followed by a decrease The increase was most marked in the warfarin treated groups The mean values of each group are visualized by the curves in Fig 3 Similar curves are obtained by the use of the median values of the groups

A comparison by analysis of variance between the individual values of the groups

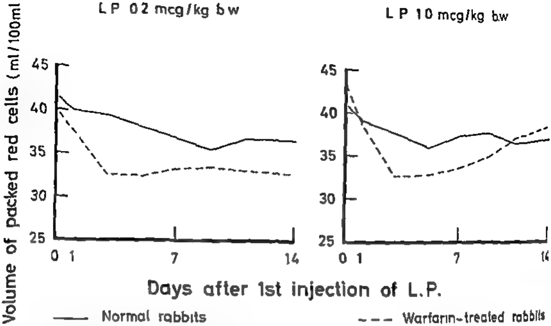

Fig 2 The volume of packed red cells (V P C) of the two groups (I and I A) receiving daily intravenous injections of endotoxin (L P) of 0 2 mcg/kg b w (left) and of the two groups (II and II A) receiving 1 0 mcg/kg b w (right)

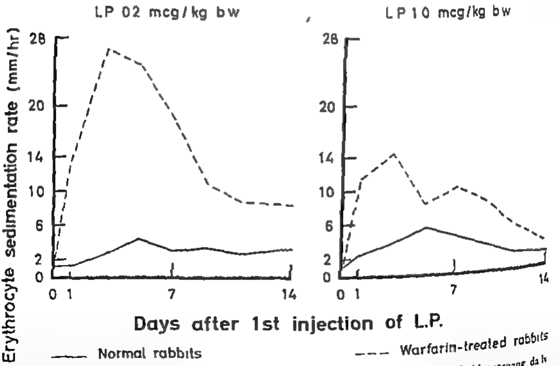

Fig 3 The erythrocyte sedimentation rate (E S R) of the two groups (I and I A) receiving daily intravenous injections of endotoxin (L P) of 0 2 mcg/kg b w (left), and of the two groups (II and II A) receiving 1 0 mcg/kg b w (right)

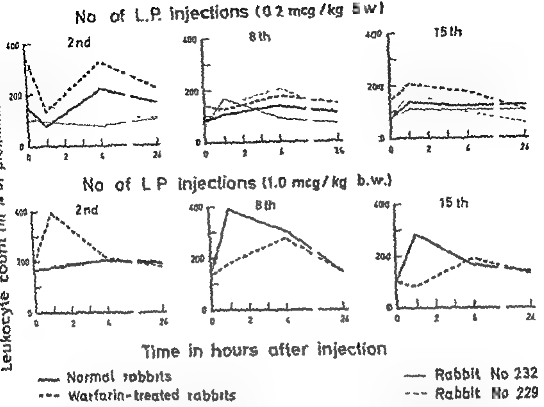

Fig 4 The total leukocyte count of the two groups (I and I A) receiving daily intravenous injections of endotoxin (L P) of 0.2 mcg/kg b w (upper), and of the two groups (II and II A) receiving 1.0 mcg/kg b w (lower) The curves for the total leukocyte count of the two rabbits, No 232 and 229 in groups I and I A, respectively, having preimmune antibodies, are drawn separately in connection with those of their groups

given equal amounts of L P showed a significant main effect of warfarin treatment In addition, a significant combined effect of warfarin treatment and the number of L P injections was found when the individual values of groups I A and I were compared

The E S R of one warfarin-treated rabbit (No 229) differed from that of the rest of its group (I A), showing only a moderate increase This rabbit did not differ from the others of its group with regard to V P C, TT% and warfarin dosage, but was one of the two rabbits having preimmune antibodies of high titre against *S typhi*

The Total Leukocyte Counts

The preimmune mean leukocyte counts of groups I A and I were 8,200 (range 4,600–13,200) and 9,940 (range 5,200–15,000), respectively, and of groups II A and II 8,300 (range 5,700–10,500) and 8,700 (range 3,000–13,200), respectively Thus the mean values are about the same, but there are considerable individual variations within each group

The leukocyte count in peripheral blood was recorded in all rabbits in connection with three of the L P injections (2nd, 8th, and 15th) during the immunization period The changes in the leukocyte count (in per cent of the preimmune count) differed considerably in the rabbits within each group, and the changes did not seem to take place simultaneously in the different rabbits Nevertheless, the mean changes in each group (Fig 4) give a fair impression of the trend of the individual changes Similar changes were found

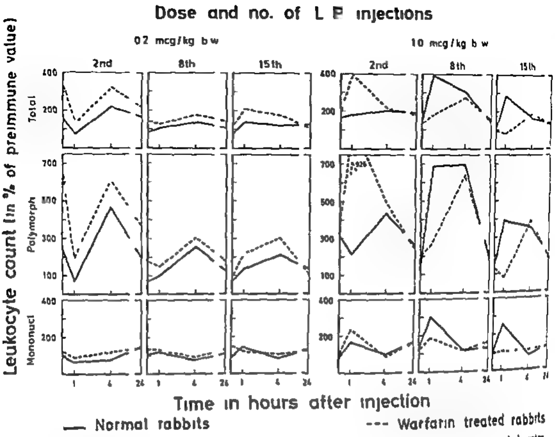

Fig 5 The total and differential leukocyte count of the two groups (I and I A) receiving daily intravenous injections of endotoxin (L P) of 0 2 mcg/kg b w (left) and of the two groups (*II* and II A) receiving 1 0 mcg/kg b w (right)

by the use of the median values of each group

The magnitude of the changes (Fig 4), especially for the lower L P dose, seems to decrease with the number of L P injections given In general, the changes seem to be more pronounced in the groups receiving the higher L P dose It is noteworthy that in the latter groups no fall in the leukocyte count was recorded 1 hr after the 2nd injection

There seems to be no difference in the leukocyte response to the lower L P dose (0 2 mcg/kg b w) between warfarin-treated (I A) and untreated (I) rabbits The two rabbits with preimmune antibodies (No 229 and No 232, one from each of the two groups), showed about equal changes and changes similar to the mean values of their groups

The changes in leukocyte count provoked by the highest L P dose (*1 0 mcg/kg b w*) seem to be different in the two groups (II A and II) with regard to the count recorded 1 hr after injection The difference, however, is not constant for the three injections Otherwise there seems to be little difference between untreated and warfarin treated rabbits.

Due to the heterogeneous individual results these impressions have not been verified by statistical analysis

The Differential Leukocyte Counts

Prior to immunization the mononuclear cells were generally found to be the most dominant of the leukocytes in peripheral blood, the mean values of groups I A and I being 57 9 per cent (range 48 0–76 5) and 62 4 per cent (range 43 5–73 5) respectively

and of groups II A and II 62.4 per cent (range 32.5–74.0) and 62.2 per cent (range 51.0–72.0), respectively.

The changes in the leukocyte count following each L.P. injection are mainly due to changes in the number of polymorphonuclear cells as visualized in Fig. 5. There seems to be no difference in polymorphonuclear cell response between the warfarin-treated and untreated rabbits receiving the lower L.P. dose (Fig. 5). However, in those receiving the higher L.P. dose, the number of polymorphonuclear cells counted 1 hr after injection in the warfarin-treated group (II A) seems to differ from that in the untreated group (II). It is noteworthy that a slight increase in the number of mononuclear cells in peripheral blood was also seen in these groups at the same time, 1 hr after each injection. Otherwise the number of mononuclear cells varies little, and in rabbits receiving the lower dose there is almost no change at all. The results in the rabbits with preimmune antibodies do not differ from those in their respective groups.

If the polymorphonuclear count 1 hr after L.P. injection in rabbits receiving the higher dose is excepted, the conclusion seems to be that there is no fundamental difference between warfarin-treated and untreated groups. Because of the heterogeneous individual results a statistical analysis of the results was not performed.

DISCUSSION

The endotoxin doses used in the present study provoked in the same rabbits a considerable biological activity with regard to pyrogenic and antibody response (15, 16). These previous reports show, moreover, that pyrogenic tolerance was obtained for the lower and for the higher endotoxin dose after the 3rd and the 5th injection, respectively. Specific antibodies were recorded 3 days after the 1st injection.

The coagulation activity, the TT%, is in the present study kept at the level required for anticoagulant treatment in man (14).

Firstly, it is noteworthy that the present endotoxin injections provoke a fall in the V.P.C. value in all groups. *Brain & Hourihane* (1) found that endotoxin injections, especially the second, which was given 24 hrs after the first, provoked in normal rabbits a marked rise in plasma haemoglobin. Their results (1) and later experiments (2) indicated, however, that heparin when given in doses preventing thrombosis, also prevented haemoglobinaemia. *Brain et al.* (2) suggested that haemolysis accompanies the endotoxin-induced thrombosis. Haemolysis as a consequence of intravascular coagulation and a subsequent haemorrhagic diathesis (25, 29) may explain the present V.P.C. fall in the untreated groups. Warfarin treatment, however, prevents the endotoxin-provoked thrombosis (32), and warfarin treatment alone gives no V.P.C. fall (14). The marked fall in V.P.C. in the warfarin-treated groups in the present study, compared with the untreated groups, is therefore probably due to another mechanism, *e.g.* that in warfarin-treated rabbits, unlike heparin-treated rabbits, endotoxin provokes haemorrhages more pronounced than the haemorrhagic diathesis caused by the intravascular coagulation. This suggestion may be supported by the finding of V.P.C. fall without haemolysis in warfarin-treated rabbits after thromboplastin infusion (27) and in rabbits without renal cortical necrosis after Liquoid injection (6).

The fall in V.P.C. coincided with an increase in E.S.R. (mm/hr), the increase being significantly higher in the warfarin-treated groups. Most probably the present E.S.R. results reflect the haematocrit changes (14), although there still is some controversy about the relation of E.S.R. to V.P.C. (23, 31). The possibility should not, however, be excluded that warfarin treatment will contribute to a further increase in an E.S.R. that is already abnormally high.

The present changes in the leukocyte count provoked by the L.P. injections are dominated by the fluctuations in the number of polymorphonuclear cells (Fig. 5). The changes in the number of mononuclear cells take

place when increased doses are given These findings are in accordance with previous reports (5, 11) Some previous results showing *only changes in the polymorphonuclear count* are probably only a reflection of the dose given The present results show that the changes in the poly*morphonuclear and mono* nuclear cell counts are not concurrent, and seem to indicate an inverse reaction or a phase displacement Others have arrived at widely varying results—usually an inverse reaction (3, 5, 11, 24)

In rabbits given the smaller dose (0 2 mcg/kg b w) in this study, the fluctuations in the leukocyte count decrease, and the reaction pattern alters, with an increasing number of L P injections It is noteworthy that *the initial drop in the leukocyte count* (1 hr after injection), which appears before the leukocytosis, disappears during the develop ment of tolerance (15) In rabbits receiving the smaller dose there was no fundamental *difference*—either *in the polymorphonuclear* or the mononuclear cell response—between warfarin treated and untreated rabbits

The explanation of the results of the higher dose (1 0 mcg/kg b w) is more complicated In nontolerant rabbits the 2nd injection was not followed (after 1 hr) by the distinct drop in leukocyte count which resulted from the injection of the lower dose The response of the polymorphonuclear cells to the same in jection is considerably more pronounced, and appears more quickly, in the warfarin treated group The response of the polymorpho nuclear cells to the later injections, or in the tolerant rabbits, ▪ inverse, ɪ ▪ the response seems more pronounced and appears more quickly in the untreated rabbits The mono nuclear cells show approximately the same response in the two groups, and the trend seems to be similar to that of the polymorphonuclear cells A different reaction pattern for groups receiving different doses seems probable (5), but in addition the results indi

rabbits This strongly indicated difference

(Fig 5) between the two groups has not been statistically verified due to the individual variations of the leukocyte count The variation, however, is not greater than that found in other reports (7, 33)

Filkins & Di Luzio (10) *found in the rat that anticoagulant* treatment with heparin prevented changes in the leukocyte count after endotoxin injections However *Good & Thomas* (12) *found in the rabbit* that heparin did not affect the endotoxin induced fluctuations of the leukocyte count in any way whatsoever the latter ▪ in accordance with the results obtained in the present study with the lower dose (0 2 mcg/kg b w) The literature is scanty, and no explanation of the different reaction pattern of polymorpho*nuclear cells in the groups receiving the* higher dose (1 0 mcg/kg b w) can be given No difference was recorded in the pyrogenic response of the two groups (15)

The fate of the polymorphonuclear cells in the complex endotoxin induced reaction is, however, still obscure—with regard to their mobilization (17, 34), their role in the trig gering mechanism of endotoxin induced intra vascular coagulation (9) and their phagocytic activity (4 18) Thus the warfarin treatment may well influence the endotoxin induced leukocyte changes through different still unknown mechanisms Further research in the field is necessary

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STUDIES ON CARCINOEMBRYONIC AND RELATED ANTIGENS IN MALIGNANT TUMOURS OF COLON-RECTUM

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Antisera against perchloric acid extract of adenocarcinomas of the large bowel were produced in four rabbits. The activity of these tumour antisera were further characterized. Antibodies were demonstrated against antigenic determinants on two proteins with β mobility, referred to as β_I and β_E. The β_I protein possessed antigens which were found to be identical to *GOLD's CEA*. β_E as well as β_I antigens were demonstrated by immunodiffusion experiments in all the twelve tumour extracts which were tested. The amount of tumour associated β proteins varied more than ten-fold in different tumour extracts. Absorption experiments showed that the β_E protein was present also in the non-tumour extract in small amounts, but no evidence was obtained that the non-tumour extract possessed β_I or CEA protein. Observations are presented which indicate that the β_I molecules, in addition to specific antigenic sites, also possess antigenic determinants which seems to be shared by the β_E molecules. The two proteins show great electrophoretic heterogeneity, but no polymorphism was detected in the β globulins by antigen-antibody crossed electrophoresis. The four antisera also showed activity against a third protein which was not further characterized.

In 1965 *Gold* & *Freedman* showed that extracts of colonic cancers possessed antigens which were absent from the corresponding normal adult tissues (3).

Identical antigens were detected in specimens of primary and metastatic carcinomas arising from the entodermally derived epithelium of the digestive system (4). It was not found in other tumours or otherwise diseased human tissues. Identical components were also found in embryonic and foetal gut, pancreas and liver in the first two trimesters of gestation. The antigens were therefore called carcinoembryonic antigens (CEA) (4).

Immunofluorescent studies have revealed that CEA are located to the cell membrane (5, 9) and cells possessing these antigens were agglutinated by anti-CEA sera (5). The position of the antigens in the ultrastructure at the surface of the neoplastic cell has been studied by electron microscopy. The antigens were found in the glycocalyx immediately adjacent to the surface membrane (6). The antigens are released into the circulation and can be detected in the serum from patients with adenocarcinomas of the colon (19). Recently it has also been found in sera from some patients with other tu-

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mous and other diseases (15, 18), and questions concerning the specificity of the methods and their antisera need critical evaluation

The monospecific anti CEA serum reacts with a β-globulin in perchloric acid extracts of colonic cancers (11, 12) Reaction against a second β-globulin has been demonstrated in some anti CEA sera by several investigators (3, 4, 7, 8, 17, 20) and activity against an α protein has been described by *v Kleist* & *Burtin* (8)

The aim of this work was further study of CEA and the complex antigen antibody systems which seem to be associated with CEA, and to characterize antisera that can be used in radioimmunoassay to detect cancer antigens in human sera (20)

MATERIALS AND METHODS

Tissue extracts of nine adenocarcinomas which were removed surgically from various parts of colon and rectum were used in this study (Table 1) A few experiments were done with extracts of liver metastases from colonic cancers obtained at necropsy (T_{10}, T_{11}, T_{12})

The diagnoses were confirmed by histological examination After removal, the tumour specimen was rinsed in tap water Normal tissues as well as necrotic lesions were removed The tissues were cut in small pieces, washed in saline and either processed immediately or frozen and kept at —22° C until processing The tissues were homogenized in a Sorvall Omni Mixer Homogenizer at 16000 rpm at 4° C for ten minutes Streptomycin was added to the homogenized tissue in an amount of 15 μg/ml to prevent bacterial growth, and extracts were made in perchloric acid as described by *Krupey* (12)

The tumour extracts were prepared separately from each tumour except in one case T_7 which was made from a pool of two different tumours (Table 1)

After preparation the extracts were concentrated in a Buchi Rotavapor at room temperature to a protein concentration of about 3–14 mg/ml (Table 1), frozen and stored at —22° C until use Extracts were prepared in a similar manner from the macroscopically normal colonic mucosa with small pieces of the muscular wall included The non tumour tissues were taken more than 3 7 cm from any visible tumour Non tumour and tumour extracts from the same patient were given identical index numbers

Immunization of Rabbits

Four rabbits were immunized with tumour extracts, each receiving five injections of 0 3-0 5 ml intradermally into the footpads at weekly intervals A booster dose was given 4 weeks after the last injection The first injection contained equal volumes of extract and *Freund's* adjuvans. Adjuvans was omitted from later injections

Tumour extract T_7 was used in two rabbits to produce antisera no 13 and 14, and extract T_7 in two other animals to produce antisera no. 57 and 58 The rabbits were bled 10 days after the last injection and further bleedings were performed at weekly intervals as long as antibody activity was unchanged The antisera were stored at —22° C until further use

Absorption of Antisera

Absorptions were done in repeated steps with incubations for 2 hrs at room temperature and overnight at 4° C The precipitates were removed by centrifugation at 2500–3000 rpm for 10–15 min The primary absorptions were done with normal plasma, and with non tumour extract obtained from the same patient as *the tumour* extract which was used for immunization. More extensive absorptions were performed with highly concentrated materials Two-fold dilutions of these materials were used in a series of absorptions or inhibition experiments

Precipitin Inhibition Studies

A modification of *Martin* & *Martin's* procedure was used (17) The peripheral wells were filled with 10 μl of tumour or non tumour extract in increasing two-fold dilutions and then filled with equal amounts of the primarily absorbed antiserum The central wells were filled with 5 μl of extract The reactions were read after 24–78 hrs of incubation at 4° C and the amount of protein able to block the antibody activity of 1 ml of antiserum was calculated

Immunoelectrophoresis and Double Immunodiffusion

Immunoelectrophoresis was performed on glass plates 8 × 8 cm in 1 per cent agarose gel and barbital buffer 0 075 M with 1 mM Ca lactate pH 8 6 Samples were run at 11–12 volts/cm for 60–90 min on plates cooled to 4–6° C

The same gel and buffer system was used in double immunodiffusion experiments Reactions were read after 24 78 hrs of incubation at 4° C.

Counter Current Electrophoresis

Counter current electrophoresis (10) was the most sensitive method to detect the α-precipitin line The same agarose buffer system as described

for immunoelectrophoresis was used Antigens placed in the cathodic well were run against antibodies in the anodic well at 5 volts/cm for 60–90 min on plates adjusted to 20° C

The diameters of the antibody and antigen wells were 4 mm and 3 mm respectively and the distance between the centres of corresponding wells 10 mm Each well was filled with 10 µl of antiserum or 5 µl of extract The plates were read immediately after the electrophoresis was run or after incubation overnight at 4° C, which markedly improved the sensitivity

Antigen-antibody Crossed Electrophoresis

A modification of *Laurell*'s method (13) was used to study the tumour associated β globulins The first run was performed as described for immunoelectrophoresis and a strip of agarose gel which contained the β-globulins was cut longitudinally in the direction of migration The strip was placed on another agarose plate which contained primarily absorbed antiserum diluted 1/40–1/100 An electric field of 11–12 volts/cm was applied at a right angle to the agarose strip and run for 4 5 hrs on plates cooled to 4–6° C

Quantitation Studies

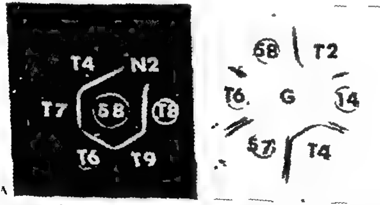
The same agarose buffer system and antisera dilutions were used to measure the concentration of tumour associated β globulins by electrophoresis in antibody-containing gel (14) Wells along the lower edge of the plate were filled with exactly 10 µl of extract and allowed to migrate into the gel in an electric field of 11–12 volts/cm for 4–5 hrs on plates cooled to 4–6° C Two-fold dilutions of tumour extract T_2 were included in each run and 1/10 of T_2 was used as reference unit.

Experiments were done in parallel by the single radial immunodiffusion method (15) Wells were cut in 1 per cent agarose gel which contained primarily absorbed antiserum in dilution 1/40–1/100 and filled with exactly 10 µl of extract The diameter of the diffusion ring was recorded after incubation at 4° C for 48 hrs and the concentration of the tumour associated β globulins calculated, based on the standard curve obtained by titration of T_2

The protein concentrations were measured by the conventional Folin-Ciocalteus phenol method (*Lowry*)

RESULTS

The four rabbits immunized with extracts of colonic carcinomas produced antibodies against tumour antigens After a primary absorption with non-tumour extract and normal human plasma as shown in Table 2, two precipitin lines were seen in double immunodiffusion experiments with tumour extracts The two lines were often more or less super



A B

Fig 1 Detection and identification of tumour antigens by double immunodiffusion A) Central well antiserum no 58 which is primarily absorbed as shown in Table 2 Peripheral wells different tumour extracts T and non tumour extract N_2 B) Central well *Gold*'s anti-CEA reference serum. Peripheral wells tumour extracts T_2 $T_?$ T_4 and primarily absorbed antisera nos 14 57 and 58 Note the precipitin lines against the tumour extracts and the lack of reaction against the non tumour extract Two precipitin lines are seen with our antisera one internal (β_I) the other external (β_E) with reference to antigen well The two lines are more or less superimposed or clearly separated Reaction of identity is seen between different tumour extracts as well as between different antisera The anti CEA reference serum gives reaction of identity with the internal precipitin line (β_I)

TABLE 1 *Perchloric Acid Extracts of Tumous (T) and Non-Tumour (N) Tissues from the Large Bowel and their Protein Concentrations (mg/ml)*

Extract No	Extract of		Protein conc	$(\beta_I + \beta_E)$ glob	$(\beta_I + \beta_E)$ glob per mg prot
T_1	Adenoca	coecum	9 3	1 4	0 15
T_2	–	rectum	3 9	10 0	2 5o
T_3	–	coecum	3 7	5 1	1 35
T_4	–	sigmoid	12 7	26 5	2 05
T_5	–	–	2 7	5 7	2 11
T_6	–	ascend	14 3	5 3	0 37
T	–	ascend } sigmoid }	4 5	5 5	1 22
T_8	–	rectum	13 3	10 9	0 82
T_9	–	coecum	9 7	10 9	1 12
N_1	Colonic mucosa		3 6	–	–
N.	–	–	4 4	–	–
N_3	–		6 8	tr	–
N_4	–		7 8	tr	–
N_5	–	–	4 0	tr	–

The amounts of tumour associated β globulin $(\beta_I + \beta_E)$ as measured by single radial immunodiffusion are given in units equal to 1/10 of T. and calculated per ml of total protein in the extract

TABLE 2 *Antibody Activity after Absorption of Tumour Antisera with Normal Plasma (NP) and Perchloric Acid Extract of Non Tumour (N) and Tumour (T) Tissues, as it Appeared in Immunodiffusion Experiments or Counter Current Electrophoresis*

	Antiserum No	One ml antiserum absorbed with NP ml	Extract No	Extract mg prot	Antibody activities after absorption β_I	β_E	α
Primary absorption	13	0 08	N_1	1 4	+ +	+	+
	14	0 08	N_3	1 4	+ +	+ +	+
	57	0 10	N_4	2 2	+ + +	+ +	+
	58	0 10	N_4	2 5	+ + +	+	+
Further absorption	58		N.	20	+ + +	(+)	—
	58		N.	40	+ + +	—	
	58		N.	90	+ + +	—	—
	57		N.	40	+ + +	(+)	
	57		N.	80	+ + +	—	
	58		T_9	4 9	(+)		—
	58		T_9	9 7	—		—

Fig 2 Immunoelectrophoresis in agarose gel The antigen well is filled with tumour extract T_4 diluted 1/4 and the antiserum trough with primarily absorbed antiserum no 57 The two precipitin lines are distinguished on the cathodic side where the β_I-line is seen slightly anodal to the β_E line On the anodic side the two lines are superimposed

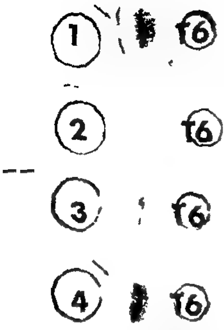


Fig 3 Counter current electrophoresis Ant gen wells are f lled with tumour extract T_8 and anti body wells nos 1–4 with antiserum no 58 which is absorbed as follows Well no 1 primarily absorbed antiserum (p abs 58) see Table 2 Well no 2 p abs 58 further abs with 4 4 mg T_4 protein Well no 3 p abs 58 further abs with 2 mg T_8 protein Well no 4 p abs 58 further abs with 2 mg N_2 protein Addition of comparable amounts of saline instead of T or N-extract had no visible effect on the α line Note the strong α line (see arrows) which is completely removed by absorption with tumour extracts (T_4 T_8) in amounts of 2–4 mg protein After absorption with 2 mg non tumour protein N_2 the α line is weaker but still visible The diffuse precipitin band closest to the antigen wells was identified as the β precipitin line or band by parallel immunodiffusion experiments

imposed No reactions were observed in corresponding experiments with non tumour extracts (Fig 1)

The two precipitin lines were demonstrated in all the nine tumour extracts shown in *Table 1* as well as in the three tumour extracts prepared from necropsy materials (T_{10} T and T_1) Antibody activity was found in all the four antisera though minor variations were observed (Table 2)

Immunoelectrophoresis showed two lines in the β region (Fig 2) The lines are referred to as β internal (β_I) and β external (β_E) according to *Burtin* & *v Kleist* (1) The internal line (β_I) closest to the antigen well was slightly more anodal than the external line (β_E) On the anodic side the two lines were superimposed

The precipitin lines which were seen with different tumour extracts showed complete identity and reactions of identity were demonstrated between the β_I line and the precipitin line given by a mono specific anti CEA serum which was kindly provided by *Gold* (Fig 1B)

An additional third line was seen by counter current electrophoresis anodal to the β lines (Fig 3) The corresponding protein was detected in the tumour extracts T_1 T_3 and T_8 but not in the other tumour or non tumour extracts shown in Table 1 Except in experiments with T_8 this line was rather weak and the corresponding protein could not be clearly identified by immunoelectrophoresis Unfortunately, lack of T_8 extracts did not allow further experiments in higher concentration *The line is referred to as the* α precipitin line, though the corresponding protein is not identified electrophoretically

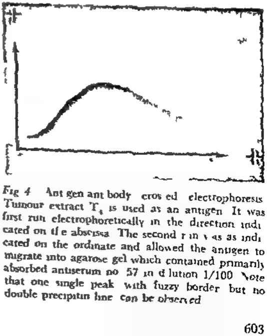


Fig 4 Antigen antibody crossed electrophoresis Tumour extract T_4 is used as an antigen It was first run electrophoretically in the direction indicated on the abscissa The second run was as indicated on the ordinate and allowed the antigen to migrate into agarose gel which contained primarily absorbed antiserum no 57 in dilution 1/100 Note that one single peak with fuzzy border but no double precipitin line can be observed

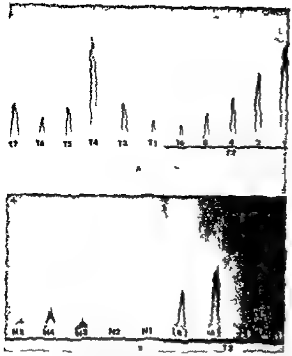


Fig 5 Quantitation of tumour associated β globulins by electrophoresis in agarose gel containing anti β_T and anti β_E antibodies Antigen wells are filled with exactly 10 µl of tumour extract as indicated Two fold dilutions of tumour extract T_2 are included as a standard The antigens are allowed to migrate into the gel which contains the primarily absorbed antiserum no 58 in dilution 1/100 Note that each tumour extract gives one single precipitin peak except T_6 where a low second peak is seen (A) This peak is probably identical to the α line which was strong with T_6 Tumour extract T_6 also shows a marked cathodal migration which is absent or weak with the other tumour extracts Note the trace reactions in non tumour extracts N_3 N_4 and N (B) No reactions are observed in the extracts N_2 and N_1.

The proteins which gave two different precipitin lines in the β region were further tested by antigen antibody crossed electrophoresis Only one single peak was seen (Fig 4) The precipitin line was fuzzy, but no distinct double line appeared in any of our experiments which were done with different antisera as well as with different tumour extracts The two β proteins showed great electrophoretic heterogeneity but no evidence of polymorphism was observed in these proteins (Fig 4)

Similar observations were done when antigen antibody crossed electrophoresis was used to estimate the concentration of these antigens (Fig 5) Except in experiments with T_6 only one single precipitin peak was seen. In this extract a low second peak was detected, possibly due to the protein which gave the α precipitin line This protein was present in higher concentration in T_6 than in the other tumour extracts Some variations were demonstrated in the electrophoretic mobility of the β globulins from different tumour extracts The difference between T_4 or T where no cathodal migration was seen and T_6 which moved about 2 cm in the cathodal direction was remarkable (Fig 5A) Only minor differences were observed between the other tumour extracts. The differences in electrophoretic mobility were confirmed by simple immunoelectrophoresis.

The antigen antibody crossed electrophoresis was therefore less suitable for quantitation purposes and the concentration of the tumour associated β antigens was also estimated by the single radial immunodiffusion method. Only one single precipitin ring was seen in these experiments except with T_6 where a second ring could be detected close to the antigen well The results obtained with the two methods did not differ much though some variations were found as expected Table 1 shows the concentration of (β_T + β_E) globulin as measured by the single radial immunodiffusion method Variations from 0 15 to 2 56 unit/mg protein were found among the various tumour extracts when 1/10 of T_2 was chosen as a unit

The antigen antibody crossed electrophoresis was more sensitive than our double immunodiffusion test Traces of tumour associated β globulins were detected by this method in the non tumour extracts N_3 N_4 and N_5 but not in extracts N_1 and N (Fig 5B)

Precipitin inhibition experiments showed that the anti tumour activity was completely inhibited by a small amount of tumour extracts while corresponding amounts of non tumour extract had no effect 5–10 mg of T_2 which had about the average amount of β

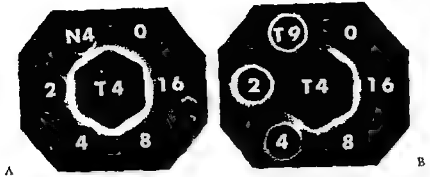


Fig 6 Precipitin inhibition experiments in agarose gel Central wells are filled with tumour extract T_4 Peripheral wells A) Antiserum no 58 which is primarily absorbed see Table 2, and equal amounts of two fold dilutions of non tumour extract N_4 as indicated by the numbers Well no 0 is filled with anti serum no 58 and equal amounts of saline B) Same as A but tumour extract T_9 is used instead of non-tumour extract N_4 The protein concentrations of the extracts are given in Table 1 Note the inhibition by tumour extract up to a dilution 1/2–1/4 No inhibition was observed by the non tumour extract The two β precipitin lines are superimposed with some dissociation seen between the wells T_9 and O

antigens thus completely inhibited the anti β activity of 1 ml primarily absorbed antiserum no 58 (Fig 6) Similar results were obtained by inhibition experiments with the other antisera and other tumour- and non-tumour extracts

Absorption experiments were done to find whether tumour associated β_I, β_E or α antigens could be detected in the non tumour extract N_2 The anti β_E activity was removed from 1 ml of the primarily absorbed anti serum no 58 by further absorption with 20–40 mg of N_2 protein, while 40–80 mg of the same protein was needed to remove completely the anti β_E activity from 1 ml anti serum no 57 which had a stronger anti β_E reaction (Fig 7) A strong anti β_I reaction could still be seen after absorption with 90 mg N protein (Table 2)

The anti α activity was completely removed from 1 ml of the primarily absorbed anti serum no 58 by further absorptions with 2–4 mg protein from tumour extract T_4, (Fig 3) while 9–18 mg protein from non tumour extract N_2 was needed to inhibit this activity completely Absorption with perchloric acid extract of normal human plasma in amounts corresponding to 500 ml plasma to 1 ml anti serum did *not visibly* reduce the anti β_E nor the anti β_I reactions

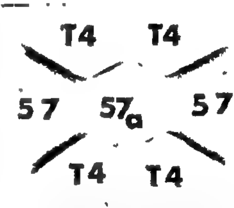


Fig 7 Double immunodiffusion after incubation for 24 hrs, showing the antibody activity of anti serum no 57 before and after absorption with non tumour protein N in high concentration Well 57 Antiserum no 57 which is primarily absorbed, see Table 2 Well 57_a Antiserum no 57 further absorbed with 40 mg N_2 protein Note the β_E precipitin line which is absent after absorption with 40 mg N_2 protein (57_a) The β_I line is still strong

DISCUSSION

The four rabbits which were immunized against perchloric acid extract of colonic adenocarcinoma produced antibodies against at least three antigens present in colonic tumours Two of the antigens were associated with proteins of β electrophoretic mobility, and could be distinguished as two separate

lines in immunodiffusion experiments, thus confirming the observations of *Gold & Kleist* and others (3, 7, 8, 17).

The β_1 line gave a reaction of complete identity with *Gold's* anti CEA serum confirming the CEA nature of the β_1 protein and also confirming the CEA specificity of our antisera. The β_E precipitin line and the corresponding antigens are likely to be identical to similar antigens described by others (3, 7, 8, 17).

In both immunodiffusion experiments and immunoelectrophoresis the two β precipitin lines were often seen separated. The β_1 line was always closest to the antigen well, and antibodies against the β_1 protein therefore had to pass through the β_E precipitin line indicating that the β_1 protein possessed antigenic sites not available on the β_E protein.

On the other hand in a number of similar experiments the two β lines were superimposed in a way which could not exclude some reaction of identity. By antigen antibody crossed electrophoresis with sera containing anti β_1 and anti β_E antibodies only one single peak was seen (Fig. 4 and Fig. 5). Nine tumour extracts with variable amounts of β globulins were included in these experiments and antisera with different anti β_1 and anti β_E activities were used (Table 2). Similar findings were obtained by single radial immunodiffusion experiments where only one precipitin ring appeared.

It is difficult to explain these observations by assuming that the two precipitin lines superimpose or that one line is too weak to be observed in all experiments. It rather suggests that the β_1 and B_E molecules have some antigenic sites in common and precipitate together. The fuzzy appearance of the precipitin line in Fig. 4 may also suggest an existence of heterogeneous antigen antibody systems which form complexes with different dissociation constants.

The finding of common antigenic sites in the two β proteins is consistent with the suggestion that these proteins may be polymeric derivatives as indicated by their molecular size (8). More likely however is the hypothesis that the two proteins have a common basic structure to which different determinant groups are conjugated (7).

It is the total amount of tumour associated β globulins with common antigenic sites which is assumed to be measured by our antigen antibody crossed electrophoresis or single radial immunodiffusion test. The concentration of CEA or β_1 antigens and the corresponding protein was not studied separately. But similar variations in CEA or CEA like antigens have been reported (17). The differences seen in the electrophoretic mobility of the β globulins from different tumours could be due to changes during processing or handling of the extracts though structural differences in the protein synthesized by individual tumours cannot be excluded. This phenomenon needs further studies. Tumour extract T_8 which had the most marked cathodal migration was prepared from a tumour localized to the ascending colon. Other tumours with the same localization did not show this electrophoretic pattern.

Tissue which was removed less than 7 cm from the tumour was included in some of our non tumour extracts. Tumour cells may be present in this tissue (3) and it was therefore not surprising that traces of tumour associated β globulins could be detected in some of our non tumour extracts by sensitive methods.

Absorption with non tumour extract in large amounts like 90 mg protein per ml of antiserum did not significantly reduce the anti β_1 or anti CEA activity which is in accordance with the observations of *Gold & Freedman* (3). Evidence has however been presented that CEA may be present in very minute amounts even in non-cancer tissue (17) and its rigorous cancer specificity has recently been the subject for further discussion (2).

The β_E antigens were not demonstrated in the non tumour extract N by double immunodiffusion experiments or antigen-antibody crossed electrophoresis. Absorption experiments did however show that small amounts of β_E antigens were present in the

non tumour extract 20–40 mg N-protein was required to remove the anti-β_E activity from 1 ml antiserum no 58, and twice this amount to remove the activity from antiserum no 57, as compared to 5–10 mg protein of tumour extract T_8 which abolished both anti β_E and anti β_1-activity These observations as well as similar observations by others (1, 17) indicate that the β_E antigens are not tumour specific, though it seems to be some evidence for the β_E-antigens to be tumour associated We could not detect β_E-antigens in normal human plasma by absorption experiments Whether the β_E antigens are present in the serum from cancer patients or patients with other diseases is not known at the present time Further work is needed to answer these questions. The use of antisera with combined anti β_1 and anti β_E activity in order to increase the sensitivity of immunoassays should be investigated There is apparently a need for screening methods with a maximum of sensitivity in addition to the more specific assay which is used to identify CEA in the sera of cancer patients (19)

The α precipitin reaction was weak, and the corresponding antigens were not studied in any detail Absorption experiments showed however that these antigens were present in non tumour as well as in tumour extracts These antigens are probably identical to the antigens described by *v Kleist* & *Burtin* (1 8)

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A POLYSACCHARIDE ANTIGEN OF AN ANAEROBIC ORAL FILAMENTOUS MICROORGANISM (*EUBACTERIUM SABURREUM*) CONTAINING HEPTOSE AND O-ACETYL AS MAIN CONSTITUENTS

3 *Some Serological Properties*

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The polysaccharide antigen PS L44 isolated from the filamentous organism L44 proved to be highly active in precipitation and complement fixation tests, and inhibited the agglutination of whole L44 bacteria in four units of antiserum L44 Sensitization of normal or tanned sheep erythrocytes was not achieved Deacetylation of the antigen with mild alkali reduced the serological activity, and disclosed another antigenic specificity as evidenced by precipitation in agar gel Under the experimental conditions used purified PS L44 was non immunogenic in rabbits Specific low molecular weight antibodies were readily obtained by immunization of rabbits with whole bacteria or isolated cell walls In addition the immunization stimulated to production of an antibody giving passive cutaneous anaphylaxis in guinea pigs and rabbits when challenged with PS L44 Cross reactivity experiments suggested that PS L44 is a type specific antigen in *Eubacterium saburreum*

A previous paper has dealt with the isolation of a polysaccharide antigen from the anaerobic oral microorganism *Eubacterium saburreum*, strain L44 (6) The antigen was released from whole bacterial cells by treatment with trypsin, and purified by fractionation of the digest on columns of Sephadex G-75 and DEAE-cellulose Approximately 90 per cent of the purified preparations were made up of heptose and O-acetyl Additional components were trace amounts of other sugars and protein

Some physical properties of the antigen have been reported in a preceding paper (7) This report presents the results of serological experiments performed with the purified untreated or deacetylated antigen

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MATERIALS AND METHODS

The isolation and cultivation of the filamentous organism, strain L44, and other oral strains included in the present study, have been described earlier (3) The polysaccharide antigen was extracted from acetone dried bacteria, and purified as reported (6) For convenience the antigen is referred to as PS L44

Deacetylation was achieved by treatment of lyophilized samples of PS L44 with 0 01 N NaOH *for 60 min at 56° C Oxidation with periodate was* carried out by mixing equal volumes of a 0 1 per cent solution of the antigen in 0 02 M phosphate buffer, pH 7 0, and 0 02 *M* sodium *meta* periodate The mixtures were incubated in the dark at 4° C overnight and dialysed against running tap water before use

Human sera were obtained from 30 individuals with varying degree of periodontal disease (12)

Serological Methods

Antisera were produced in rabbits by intravenous injections of washed bacteria or undigested cell walls (5) Rabbits were also injected with purified PS L44 in sterile saline or mixed with an equal volume of Freund's complete adjuvans

Gel filtration of antiserum was performed at 4° C on a 94 cm high column of Sephadex G 200, internal diameter 2 4 cm, with 0 1 M tris HCl, pH 8 0, containing 1 M sodium chloride, as eluant

The methods for *indirect haemagglutination* using normal or tanned (tannic acid 1 40,000) sheep erythrocytes and *complement fixation* have been reported in (5) In complement fixation tests two 100 per cent lytic units of complement and two units of amboceptor were used Tubes with 0 1 ml of serum dilution, 0 1 ml of antigen and 0 2 ml of complement were incubated overnight at 4° C after which 0 2 ml of a 1 per cent suspension of sensitized sheep erythrocytes was added

Ring test precipitation and *double diffusion* in agar were performed as described in (6)

Quantitative precipitation was carried out essentially as described in (10) 0 3 ml of undiluted serum was mixed with an equal volume of antigen solution, placed at 37° C for 1 hr and in the refrigerator for [illegible] days Antibody N was determined by the micro Kjeldahl procedure (10) Samples were digested for 6 hr in the same tubes which had been used for precipitation and washings

Inhibition of agglutination Two fold dilutions of PS L44 were prepared in 0 1 ml volumes in saline To each tube was added 0 5 ml of a dilution of antiserum L44 containing 4 agglutinating units Following incubation at room temperature for 60 min 2 drops of a saline suspension of washed L44 bacteria, standardized to an optical density at 600 mμ of 2 0, were added to each tube The agglutination was read after incubation at 37° C overnight

Passive cutaneous anaphylaxis (PCA) reactions were produced in white guinea pigs and in albino rabbits 0 2 ml of undiluted rabbit antiserum [illegible] normal serum was injected intradermally on the shaved abdomen 5 hr before challenge intravenously with 1 mg PS L44 mixed with 5 mg of Evans blue in 0 5 ml of saline

Immunoelectrophoresis was carried out as described in (7) with a veronal buffer pH 8 7 ionic strength 0 06

RESULTS

Serological Reactivity

The serological activity of PS L44 was examined against antisera to whole L44 bacterial cells

Low doses of antigen were active in precipitation and complement fixation tests and completely inhibited the agglutination of L44 bacteria in homologous antisera (Table 1) By chessboard titration of dilutions of antigen

TABLE 1 *Serological Reactivity of Untreated and Deacetylated (0 01 N NaOH 56° C, 60 min) Polysaccharide Antigen PS L44*

Treatment	Precipitating activity (μg/ml)*	Complement binding activity (μg/ml)§	Minimal dose inhibiting agglutination (μg)†
None	0 49	0 078	1 56
Deacetylation	3 90	0 625	>100

* Least concentration (two-fold dilution) of antigen giving positive ring test

§ Least concentration (two-fold dilution) of antigen giving maximum serum titres

† Bacterial agglutination, 4 agglutinating units of antiserum

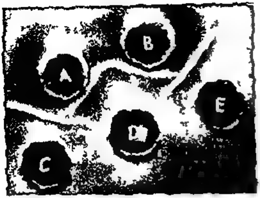

Fig 1 Agar precipitation bands produced by untreated polysaccharide antigen PS L44 0 1 mg/ml (wells C and E) and by deacetylated PS L44 0 5 mg/ml (well D) against antiserum L44 (wells A and B)

and antiserum maximal serum titres in complement fixation tests, 1/320 to 1/640 were obtained with doses of PS L44 from 0 25 to 0 0078 μg However, detectable complement fixation was observed with amounts of antigen as low as 0 00098 μg PS L44 did not sensitize normal or tanned sheep erythrocytes to agglutination in L44 antiserum

The serological activity of PS L44 in precipitation and complement fixation tests decreased upon deacetylation and the deacetylated antigen did not inhibit bacterial agglutination (Table 1) As shown by double diffusion in agar (Fig 1) deacetylation of PS L44 by mild alkali altered its serological specificity The precipitation line given by deacetylated PS L44 did not coalesce with the line produced by the untreated polysaccharide However the two lines did not cross With one antiserum L44 containing high amounts of antibodies to deacetylated polysaccharide there was a partial coalescence between the lines with formation of a spur

In another experiment a sample of antiserum L44 which had been absorbed with a surplus of deacetylated PS L44 was mixed with untreated PS L44 A heavy precipitate was obtained The washed precipitate was heated at 100° C for 2 min to denature the antibodies bound in the precipitate and a portion of it was deacetylated by treatment with sodium hydroxide This deacetylated precipitate on agar precipitation against antiserum L44 gave one line which coalesced partially with the line given by the heated untreated precipitate, but which coalesced completely with the line given by a sample of deacetylated PS L44

The quantitative precipitin curve is shown in Fig 2 As antigen was used untreated PS L44 The rabbit antiserum employed contained large amounts of precipitating antibodies to untreated PS L44 and minute but clearly detectable antibodies to the deacetylated antigen Neither antibody nor antigen were detected when the original supernatants corresponding to the zone of maximal precipitation were tested by double diffusion in agar against untreated or deacetylated PS L44 and against antiserum L44, respectively Antibodies to deacetylated PS L44 were found only in the supernatants corresponding to the extreme antibody excess zone

Oxidation with periodate destroyed the serological reactivity of both untreated and deacetylated PS L44

Immunogenicity

Antibodies against the polysaccharide antigen were readily obtained from rabbits by immunization with whole microbes or undigested cell walls Most sera were low titered with respect to the deacetylated antigen By fractionation of antiserum L44 on the Sephadex G 200 column the usual protein peaks

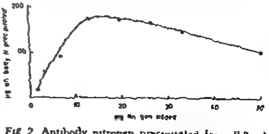

Fig 2 Antibody nitrogen precipitated from 0 3 ml of antiserum L44 by various amounts of polysaccharide antigen PS L44

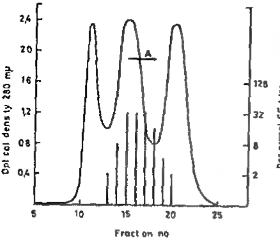

Fig 3 Fractionation of 2 ml antiserum L44 on a column (2 4 × 94 cm) of Sephadex G 200 A—fractions reacting by precipitation in agar gel against polysaccharide antigen PS L44 Complement fixation (CF) titres against PS L44 given by bars Fractions of 10 ml were collected

were observed (Fig 3) The antibody activity against PS L44, as measured by agar precipitation and complement fixation, was associated with the second peak Antibodies in low titres against the deacetylated polysaccharide were demonstrated in fractions 16 to 18 (second peak) by means of the complement fixation test

The nature of the precipitating antibodies was also studied by electrophoresis and immunodiffusion An immunoprecipitate was produced by mixing 0 1 ml of a 0 01 per cent solution of PS L44 with 1 ml of anti serum L44 The washed precipitate was partly dissolved in a great excess of antigen and examined by immunoelectrophoresis against goat anti rabbit serum A distinct IgG band but no other bands was demonstrated

Undiluted antiserum L44 gave positive PCA reactions in guinea pigs with areas of blueing up to 20 mm diameter Normal rabbit serum produced no blueing In rabbits the reactions were weaker (diameter 10–14 mm) (Fig 4) The reactions appeared within 20 min after challenge and reached maximum intensity in about 1 hr The lesions were slightly oedematous but without haemorrhages

Immunization of rabbits was also tried with purified PS L44, or with PS L44 mixed with Freund s complete adjuvans While purified polysaccharide antigen was given intravenously, the mixtures of PS L44 and adjuvans were injected intramuscularly or subcutaneously at different sites Varying doses (0 25 5 mg) of antigen and different schedules of immunization were used Antibodies to PS L44 were not detected in any instances by ring test precipitation or in complement fixation tests, or by PCA reaction in rabbits

Cross Reactivity

Ten other oral strains of *Eubacterium saburreum* were digested with trypsin as described (5), and the extracts examined by ring test precipitation and by double diffusion in agar against antiserum L44 All extracts gave a positive ring test Six of the ten strains produced the specific *PS L44 line* while the remaining four strains *did not*

Fig 4 PCA reactions in a rabbit Photograph taken 1 hr after intravenous challenge with 1 mg PS L44 mixed with 5 mg Evans blue 0? ml volumes of antiserum L44 were given intradermally as indicated by arrows 5 hr before challenge X—artificial haemorrhage

However, these four strains produced a similar major precipitinogen, detectable in high dilutions of the extracts against their homologous antisera. Treatment of the extracts from the four strains with mild alkali altered the specificity of the major precipitinogen as indicated by the immunodiffusion pattern in the same way as described for PS L44.

Finally a panel of 30 human sera were examined by agar precipitation and in complement fixation tests against dilutions of PS L44. Precipitating or complement binding antibodies were not detected.

DISCUSSION

The O acetyl containing homopolysaccharide antigen PS L44 isolated from the Gram variable oral microorganism *Eubacterium saburreum* proved to be highly active in precipitation and complement fixation tests. Deacetylation of the antigen by mild alkali was followed by an 8 fold decrease in serological activity. Furthermore the deacetylation disclosed another antigenic specificity, as evidenced by double diffusion in agar. The results of the serological examinations carried out on the immunoprecipitate obtained by mixing untreated PS L44 with antiserum L44 absorbed with deacetylated PS L44 showed that both antigenic determinants are part of one and the same macromolecular complex.

Deacetylated PS L44 did not combine with antibodies to untreated antigen. On the other hand untreated PS L44 was able to absorb antibodies to deacetylated PS L44. Apparently the immunodeterminant of the alkali degraded polysaccharide antigen is not buried in the isolated native polysaccharide but exposed and accessible for combination with specific antibodies. By agar precipitation the reaction between this particular determinant and antibody is obscured by the heavy precipitation line formed between untreated PS L44 and its homologous antibody.

The significance of O acetyl for antigenic specificity was first observed in pneumococcal Type I capsular polysaccharide (1), and was later observed with most pneumococcal polysaccharides (8). If present O acetyl groups seem to be of importance also for the serological specificity of polysaccharide antigens isolated from Gram negative bacteria (11 15 9).

The purified polysaccharide acted like a hapten when used for immunization of rabbits. Since relatively low doses of the antigen were used in the immunization experiments it is unlikely that the failure to induce production of antibody in these rabbits was due to immunological paralysis.

The detectable specific precipitating and complement binding antibodies produced in rabbits in response to immunization with whole microbes belonged to the IgG class of immunoglobulins. The nature of the heterocytotrophic anaphylactic antibodies and the antibodies eliciting PCA reactions in other rabbits was not examined. Heterocytotrophic rabbit antibodies are known to be IgG immunoglobulins (14), whereas antibodies produced in the rabbit capable of inducing increased vascular permeability in the skin of other rabbits may be IgG antibodies (2), or belong to a class of immunoglobulins distinct from IgG, IgA and IgM (13).

The presence of PS L44 in some but not all strains of *Eubacterium saburreum* suggests that it is a type antigen. It is a major antigen as evidenced by precipitation and by the capacity to inhibit bacterial agglutination in L44 antiserum, superficially located in the bacterial cell (7). The finding of highly active alkali labile precipitinogens in the PS L44 negative *Eubacterium saburreum* strains, all of which have heptose in the undigested cell envelope (4), indicates that these strains contain type antigens with a chemical composition similar to PS L44.

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DIRECT AND INDIRECT IMMUNOFLUORESCENCE OF UNFIXED AND FIXED MYCOPLASMA COLONIES

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This report provides a description of indirect immunofluorescence of unfixed mycoplasma colonies ("indirect epifluorescence") Comparison was made between direct and indirect immunofluorescence using unfixed and hot water fixed colonies 7 mycoplasma strains were used, 6 of canine and 1 of human *(M pneumoniae)* origin It is concluded that indirect immunofluorescence of unfixed colonies is a useful serological method of identification It is more sensitive than and equally specific as direct immunofluorescence and in addition does not give the background fluorescence associated with that method It is also concluded that direct and indirect immunofluorescence of hot water fixed colonies is too unspecific for the identification of canine mycoplasma species *M pneumoniae* appears to differ in this respect as its antigenic specificity is apparently better retained during hot water fixation

In spite of the fact that immunofluorescence is a simple and rapid method it has only been employed to a rather limited extend in the serological identification and classification of mycoplasmas

Immunofluorescence has primarily been used in the identification of mycoplasmas *fixed to slides* either by chemical fixation of mycoplasmas grown in liquid medium and cell cultures (2, 7, 11, 15, 20) or by hot water fixation of agar colonies (6) The latter method of fixation has been employed with indirect immunofluorescence of *M pneumoniae* (5, 12, 13) and with both direct and indirect immunofluorescence of *M hominis* (7 16) and *M arthritidis* (7)

Unfixed colonies of mycoplasmas grown on agar plates have been identified using direct immunofluorescence, the reaction being read in a fluorescent microscope with incident illumination (1, 3, 9) In addition, indirect immunofluorescence of unfixed colonies has been used for serological classification of T-mycoplasmas (4)

The purpose of the present study was

a) to give a more detailed description of indirect immunofluorescence of unfixed colonies on agar
b) to compare the direct and indirect methods of immunofluorescence using unfixed colonies, and
c) to compare the specificity of immunofluorescence of fixed and unfixed colonies in both direct and indirect immunofluorescence

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MATERIALS AND METHODS

Mycoplasma strains 6 strains of canine source were used, viz the type strains of *M spumans* (PG 13), *M canis* (PG 14), *M maculosum* (PG 15) and *M edwardii* (PG 24), (10, 19) together with 2 as yet unclassified strains (11 b and 12 b) isolated from the trachea and the parietal pleura of a 3-month-old dog (*Rosendal* to be published) In addition, the Mac strain of *M pneumoniae* was examined

Preparation of antisera Antigens used for the immunization of rabbits were prepared from cultures grown in liquid medium consisting of rabbit meat infusion broth supplemented with 15 per cent rabbit serum 10 per cent yeast extract, 0 002 per cent DNA (Sigma), thallium acetate 1 1000, and penicillin 1,000 i u /ml

Albino rabbits were immunized 5 times at intervals of 48 hours using increasing amounts of antigen mixed with equal parts of complete Freund adjuvant A booster dose was given 6 weeks later and, after another 10 days, the rabbits were exanguinated Sera were stored at −20°C

Conjugation of serum The procedure used was that described by *Nairn* (17) Globulin was precipitated by ...
sulphate Co...
cyanate (FI...
5370) was performed at 25°C and pH 9 5 in phosphate buffer adding one part of FITC to 80 ...

PBS

The molecular relationship between FITC and protein (F/P ratio) was calculated by estimating the protein concentration of the conjugate (C_P) according to the method of *Lowry* (14) and measuring the optical density of the conjugate diluted to contain 0 5 mg protein per ml ($OD_{0.5}$) in a Zeiss photometer at a wavelength of 495 nm The molecular relationship was calculated according to the following formula

$$F/P = 2.8 \frac{OD_{0.5}}{C_P} \quad (18)$$

For indirect immunofluorescence a commercial horse anti rabbit immunoglobulin (PK 17 2-F3) conjugated with FITC was used (Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandsche Roode Kruis)

Colonies for immunofluorescence The mycoplasmas were grown on heart infusion agar (Difco) supplemented with 10 per cent yeast extract 20 per cent horse serum, 0 002 per cent DNA (Sigma), thallium acetate 1 1000, and penicillin 500 i u / ml The plates were inoculated with approx 10^3 c f u and incubated aerobically for 4 days at 37°C

Unfixed agar colonies Agar blocks cut in various geometric figures and approximately ½ x ½ cm in size were used for immunofluorescence of unfixed agar colonies The blocks were placed on slides with the colony side upwards

Hot water fixed colonies Hot water fixation of mycoplasma colonies was performed as described by *Clark et al* (6), with one modification. Slides with the agar blocks facing downwards were placed at an angle of 45° in a 100 ml beaker containing hot distilled water at 80°C. The modification consisted in maintaining the temperature of the water constant at 80°C until the blocks fell off, which usually occurred after one half to one minute The slides were then immediately washed in distilled water at 80°C and airdried

Direct immunofluorescence With the direct method a drop of conjugate or conjugate dilution was placed on the agar blocks These were then incubated for 30 minutes in a moist chamber at 20°C A preliminary study estimating specific fluorescence and background fluorescence had shown that incubation at 20° C was preferable to ...

ten minutes in a test tube placed in a slowly revolving automatic shaker (HETO, Birkerød, Denmark) Previous study had shown that two periods of ten minutes washing each gave specific fluorescence with a minimum of background fluorescence The blocks were then placed on slides again

Direct immunofluorescence of hot water fixed colonies was carried out according to the same principles as those used for unfixed agar colonies. After the last wash with buffer the slides were rinsed for one half minute in distilled water thereby preventing precipitation of salt crystals during drying

Indirect immunofluorescence Immunofluorescence using the indirect technique was carried out as described by *Black* (4) The blocks were first incubated with a drop of serum dilution for 30 minutes at 20°C in a moist chamber The blocks were washed twice for ten minutes with PBS pH 7 1 in a test tube and then transferred to the slides. A drop of conjugated antirabbit globulin was placed on each block and incubated for another 30 minutes at 20°C After washing twice in PBS pH 7 1, the blocks were finally placed on slides and read The incubation temperature and time together with the washing procedure were the results of preliminary studies as described under direct immunofluorescence

Indirect immunofluorescence of hot water fixed colonies was carried out in the same way as that used for unfixed colonies After the last wash in buffer the slides were rinsed for one half minute in distilled water

Fluorescence microscopy Fluorescence was studied using a Zeiss Standard (RA) microscope equipped with an Osram HBO mercury lamp In addition the microscope was fitted with an incident illumination attachment which prevented the passage of exciting light through the agar and slide Fluorescence was estimated with a BG 12/4 filter in exciting light and with a No 47 barrier filter in emission light The intensity of fluorescence was judged according to the following scale 0 +, + + and + + +, i e none, weak, moderate and strong fluorescence

EXPERIMENTS AND RESULTS

Comparison of Direct and Indirect Immunofluorescence of Unfixed Colonies

In order to compare direct and indirect immunofluorescence of unfixed colonies, the sensitivity and specificity of both methods were studied Sensitivity was examined using homologous titration The optimal dilution i e the highest dilution giving strong fluorescence was determined for each conjugate Specificity of the direct and indirect technique was examined in cross titrations Conjugates were used undiluted and in the optimal dilution the antisera diluted 1 10 and in the optimal dilution Undiluted antiserum was not used because it as a rule gave a weaker fluorescence than dilution 1 10 In case of heterologous cross reaction growth inhibition test using the method of *Clyde* (8) was undertaken for comparison

As seen in Table 1 the indirect immunofluorescence method generally possessed a greater sensitivity than the direct method since the optimal dilution of serum with a single exception (*M spumans* antiserum) was 2–8 times higher than the corresponding optimal dilution of conjugate

Cross titration with direct immunofluorescence of unfixed colonies showed a slight cross reaction between *M spumans* and strain 12 b, and a complete cross reaction between *M edwardii* and strain 11 b (Table 2). Corresponding cross reactions were found by indirect immunofluorescence of unfixed colonies (Table 2)

No cross reactions between the type strains of the four canine mycoplasma species tested with either method were found

Growth inhibition studies showed a partial inhibition of strain 12 b with *M spumans* antiserum, whereas strain 12 b antiserum did not inhibit *M. spumans* Between *M edwardii* and strain 11 b a double cross reaction with a complete zone of inhibition was seen

Immunofluorescence of Fixed and Unfixed Colonies

To compare the specificity of the immunofluorescence of fixed and unfixed colonies, the same titrations as those shown in Table 2 were repeated with hot water fixed colonies

Direct immunofluorescence of hot water fixed colonies showed a high degree of non-specificity compared to that of unfixed colonies since it proved impossible to differentiate fluorescence in homologous and heterologous titrations Indirect immunofluorescence was found to be less specific when fixed rather than unfixed colonies were used

TABLE 1 *Optimal Homologous Conjugate Dilutions and Serum Dilutions in Direct and Indirect Immunofluorescence together with the F/P Ratio of the Conjugates*

Antiserum against	Optimal conjugate dilution in direct immunofluorescence	Optimal serum dilution in indirect immunofluorescence	F/P ratio
M spumans (PG 13	1 40	1 40	2 8
M canis (PG 14)	1 40	1 160	4 1
M maculosum (PG 15)	1 40	1 80	5 6
M edwardii (PG 24)	1 20	1 160	5 0
Strain 11 b	1 10	1 20	2 2
Strain 12 b	1 20	1 160	6 0

TABLE 2 *Cross Titrations in Direct and Indirect Immunofluorescence Using Unfixed Agar Colonies*

Direct immunofluorescence	Conjugates and antisera against PG 13 undil	PG 13 dil 1 40	PG 14 undil	PG 14 dil 1 40	PG 15 undil	PG 15 dil 1 40	PG 24 undil	PG 24 dil 1 20	11 b undil	11 b dil 1 10	12 b undil	12 b dil 1 20
Mycoplasma strains												
M spumans (PG 13)	+ + +	+ + +	0	0	0	0	0	0	0	0	0	0
M canis (PG 14)	0	0	+ + +	+ + +	0	0	0	0	0	0	0	0
M maculosum (PG 15)	0	0	0	0	+ + +	+ + +	0	0	0	0	0	0
M edwardii (PG 24)	0	0	0	0	0	0	+ + +	+ + +	+ + +	+ +	0	0
Strain 11 b	0	0	0	0	0	0	+ + +	+ +	+ + +	+ + +	0	0
Strain 12 b	+	0	0	0	0	0	0	0	0	0	+ + +	+ + +
Indirect immunofluorescence	Dilutions 1 10	1 40	1 10	1 160	1 10	1 80	1 10	1 160	1 10	1 20	1 10	1 160
Mycoplasma strains												
M spumans (PG 13)	+ + +	+ + +	0	0	0	0	0	0	0	0	0	0
M canis (PG 14)	0	0	+ + +	+ + +	0	0	0	0	0	0	0	0
M maculosum (PG 15)	0	0	0	0	+ + +	+ + +	0	0	0	0	0	0
M edwardii (PG 24)	0	0	0	0	0	0	+ + +	+ + +	+ + +	+ + +	0	0
Strain 11 b	0	0	0	0	0	0	+ + +	+ + +	+ + +	+ + +	0	0
Strain 12 b	+	0	0	0	0	0	0	0	0	0	+ + +	+ + +

TABLE 3 *Cross Titrations in Direct and Indirect Immunofluorescence Using Hot Water Fixed Colonies*

Direct immunofluorescence	Conjugates and antisera against											
	PG 13		PG 14		PG 15		PG 24		11 b		12 b	
Mycoplasma strains	undil	dil 1 40	undil	dil 1 40	undil	dil 1 40	undil	dil 1 20	undil	dil 1 10	undil	dil 1 20
M spumans (PG 13)	+ + +	0	+ + +	0	+ + +	0	+ +	0	+ + +	+	+ + +	0
M canis (PG 14)	+ + +	0	+ + +	+	+ + +	+	+ + +	+ +	+ + +	+	+ + +	+ +
M maculosum (PG 15)	+ +	0	+ + +	0	+ + +	0	+ + +	0	+ + +	+	+ + +	0
M eduardii (PG 24)	+ + +	0	+ + +	0	+ + +	0	+ + +	+ +	+ + +	+ +	+ + +	+ +
Strain 11 b	+ + +	0	+ + +	0	+ + +	0	+ + +	+ +	+ + +	+ +	+ + +	+ +
Strain 12 b	+ + +	0	+ + +	0	+ + +	0	+ + +	0	+ + +	+	+ + +	+

Indirect immunofluorescence	Dilutions											
Mycoplasma strains	1 10	1 40	1 10	1 160	1 10	1 80	1 10	1 160	1 10	1 20	1 10	1 160
M spumans (PG 13)	+ + +	+ + +	+	0	+	0	0	0	+	+	+ + +	+
M canis (PG 14)	+ +	+	+ + +	+ +	+	0	+	+	+ +	+ +	+ +	+ +
M maculosum (PG 15)	+ + +	+	+ +	+	+ + +	+ + +	+	+	+	+	+	+
M eduardii (PG 24)	+ +	+ +	+ + +	+ +	+	+	+ + +	+ + +	+ + +	+ + +	+ +	+
Strain 11 b	+ +	+ +	+ + +	+ +	+ +	+	+ + +	+ + +	+ + +	+ + +	+	+
Strain 12 b	+ + +	+ + +	+	+	+ +	+	+ + +	0	0	0	+ + +	+ +

TABLE 4 *Homologous and Heterologous Titrations by Indirect Immunofluorescence of Hot Water Fixed* M pneumoniae *Colonies*

Dilutions	Antiserum against					Normal rabbit serum
	M pneumoniae (Mac strain)	*M spumans* (PG 13)	*M canis* (PG 14)	*M maculosum* (PG 15)	*M edwardii* (PG 24)	
1 10	+ + +	+ +	+	+	0	+
1 20	+ + +	+ +	[illegible]	0	0	0
1 40	+ + +	0	[illegible]	0	0	NT
1 80	+ + +	0	[illegible]	0	[illegible]	NT
1 160	+ + +	0	[illegible]	0	0	NT

NT – not tested

but still more sensitive than the direct one (Table 3)

Since indirect immunofluorescence of hot water fixed colonies has been used for the identification of *M pneumoniae*, the specificity of this method was studied in relationship to the latter organism (Table 4) Hot water fixed colonies of *M pneumoniae* demonstrated non specific fluorescence with heterologous serum in dilution 1 10 and 1 20, but not in higher dilutions

DISCUSSION

The direct immunofluorescence method on whole agar plates as described by *Del Giudice et al* (9) requires rather large amounts of conjugate (or dilutions of conjugate), whereas the use of agar blocks (1) provides an essential saving of conjugate as one drop is sufficient for each block

Direct immunofluorescence with undiluted conjugate gives a background fluorescence from the agar blocks (Fig 1) It is stated (17) that an F/P ratio substantially over 5 will increase background fluorescence, while and F/P ratio less than 2 produces a too weak specific fluorescence In the present study no difference in the background fluorescence was noted between the conjugates employed, even though the F/P ratios of these conjugates ranged from 2 2 to 6 0

Indirect immunofluorescence does not give the background fluorescence of the direct method and has furthermore the advantage that only one conjugated serum is required i e a commercially available antirabbit globulin

The fluorescence of unfixed agar colonies is quite uniform During washing in buffer peripheral areas of the colony can be partly rinsed away, especially where the mycoplasmas show abundant surface growth, as for example *M edwardii* This, however, does not imply a reduction in the intensity of fluorescence (Fig 1)

As shown by comparison of direct and indirect immunofluorescence of unfixed colonies on agar blocks, the indirect method is more sensitive and it possesses the same degree of specificity (Table 2) This makes indirect immunofluorescence well suited for identification and classification of mycoplasmas

As a rule, good fixation was obtained with the hot water technique and only little residual agar was left on the glass slides but only about 20 per cent of the *M spumans* colonies from an agar block could be fixed In addition, staining of hot water fixed colonies often results in fluorescence of only the central portion of the colony (Fig 2) A much more important disadvantage is that hot water fixation may reduce the specificity of immunofluorescence to such a degree that the method is unsuited for the identification of the canine mycoplasmas (Table 3)

Chanock et al (5) were aware that a certain denaturation of antigens could take place in mycoplasma colonies during hot

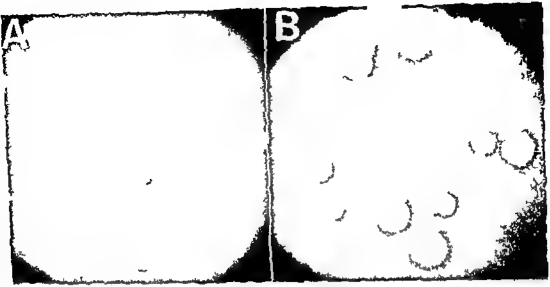

Fig 1 Unfixed agar colonies of *M edurardi* (A) Direct immunofluorescence with undiluted homologous conjugate (B) Indirect immunofluorescence with homologous serum diluted 1 160

water fixation *Lind* (12 13) considers antibody titres less than 1 40 in indirect immunofluorescence of hot water fixed *M pneumoniae* colonies as nonspecific Our experiments have confirmed this, since non specific fluorescence was seen with heterologous sera in dilutions of 1 10 and 1 20 Fluorescence became however specific in serum dilution 1 40 and higher In corresponding experiments with canine mycoplasmas greater specificity was not obtained when serum was diluted The difference can perhaps be due to a greater thermolability of the antigen from the canine mycoplasmas

On the basis of the present investigations it can be concluded that indirect immuno

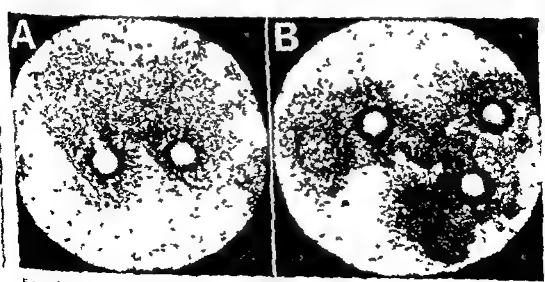

Fig Hot water fixed colonies of *M edurardi* (A Indirect immunofluorescence with homologous serum diluted 1 160 B Indirect immunofluorescence with *M canis* antiserum diluted 1 160

fluorescence of unfixed mycoplasma colonies is well suited for rapid serological identification of mycoplasmas because

a) it is more sensitive and just as specific as the direct method,
b) it can be applied as soon as growth of mycoplasma colonies on agar has been obtained,
c) there is no background fluorescence,
d) the agar block technique has the advantage of economy with respect to the amount of serum required,
e) only one commercially available conjugated serum is necessary

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BRIEF REPORT

GLIDING AND TWITCHING MOTILITY OF BACTERIA UNAFFECTED BY CYTOCHALASIN B

Jørgen Henrichsen

The cytochalasins, a group of mould metabolites, inhibit movement and cytoplasmic cleavage of cells in culture, *e g* mouse fibroblasts, as demonstrated by *Carter* in 1967 (2) Cytochalasin B is commercially available from Imperial Chemical Industries Limited In 1971, *Wessells et al* (10) stated that cytochalasin B reversibly inhibits the contractile microfilament machinery of many cell types and they furthermore predicted 'that sensitivity to the drug implies presence of some type of contractile microfilament system' They listed 14 different biological processes—*e g* single cell movement of *L* and heart fibroblasts, cytoplasmic streaming of *Nitella*, blood clot retraction and smooth muscle contraction—as being sensitive to cytochalasin B and 11 processes as being insensitive to the drug—*e g* sperm tail function, ciliary function and *Escherichia coli* flagellar movement (10) *Allison et al* (1) in 1971 reported that the processes of cell movements, ruffled membrane formation phagocytosis pinocytosis and directional movement of pinosomes all were reversibly inhibited in mouse peritoneal macrophages by exposure to 10 μg/ml of cytochalasin B and they concluded that the system of seemingly actin like microfilaments found in the peripheral cytoplasm of the macrophages plays a role in these processes

Bacteria may spread *on the surface* of solid substrates by means of six different types of surface translocation *vi* swarming, swimming gliding, twitching sliding and darting (5) Swarming and swimming are due to flagellar activity and sliding and darting to forces generated by cell division during growth (5) The mechanisms of gliding and twitching motility on the other hand, are virtually unknown, but *Pate* & *Ordal* (7) suggested that peripheral fibrils associated with the outer unit membrane might play a role in the gliding motility of a strain of *Chondrococcus columnaris*, and *Schmidt Lorenz* & *Kuhlwein* (9) went much further by stating that intracytoplasmic microfilaments, as seen in the electron microscope, of different species of myxobacteria represent contractile locomotor organelles The presence of fimbriae is most likely a necessary condition for twitching motility (6), but this does not explain the mechanism of movement

It was therefore deemed highly important to examine the effects of cytochalasin B on gliding motility and to include also in the study some bacterial strains exhibiting twitching motility

Due to the poor solubility of cytochalasin B in water a stock solution (0 1 per cent) was made in dimethyl sulphoxide (DMSO) Cytophaga agar plates—made of 0 05 per cent Bacto tryptone, 0 05 per cent Bacto yeast extract and 1 0 per cent Bacto agar—were used 1) with cytochalasin B added to give final concentrations of 1 μg/ml (0 1 per cent DMSO), 10 μg/ml (1 0 per cent DMSO) and 50 μg/ml (5 0 per cent DMSO), 2) without cytochalasin B but with the same concentrations of DMSO and 3) with neither cytochalasin B nor DMSO Control experiments using the described inhibition by the drug of cytoplasmic streaming in cells of *Nitella* were also performed Three gliding strains, *viz* one strain of *Cytophaga succinicans* and two unnamed strains of *Cytophaga* and four twitching strains of *Acinetobacter calcoaceticus* were studied by agar plate microscopy, ... cytochalasin B There was no difference in growth and spreading on plates containing cytochalasin B compared with the plates containing corresponding amounts of DMSO, but without the drug In fact, gliding motility was seen in the microscope in two of the three strains of *Cytophaga* and twitching motility in three of the four strains of *Acine*

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tobacter calcoaceticus on plates with 50 µg/ml of cytochalasin B, although growth was greatly inhibited on these and the plates with the corresponding amounts of DMSO alone (5.0 per cent). The remaining two strains did not grow at all on these plates. Neither growth nor the two kinds of motility were inhibited on any of the plates with 10 µg/ml of cytochalasin B compared with the control plates without the drug and DMSO. Accordingly, it may be concluded firstly that gliding and twitching motility of bacteria is not affected by cytochalasin B, and secondly that 5 per cent DMSO definitely inhibits growth of the bacterial species examined whereas DMSO in concentrations of 1 per cent or less exerts no appreciable influence on growth.

The control experiments performed with the drug showed that it did inhibit the cytoplasmic streaming in cells of *Nitella*. Both in water and in a 1 per cent solution of DMSO the rate of this streaming was 35–40 µ per sec but dropped quickly after transfer to a solution containing 10 µg/ml of cytochalasin B (and 1 per cent DMSO), and within an hour values of 1.5 µ per sec were registered. When the cells were transferred back to a solution of 1 per cent DMSO without cytochalasin B the rate of the cytoplasmic streaming within an hour was 20–30 µ per sec, i.e. it returned to almost normal pre-experimental levels. However, the cells always appeared somewhat changed morphologically after exposure to DMSO solutions of 1 per cent or more, which probably means that DMSO is not entirely harmless to these cells in the concentrations mentioned. Quite incidentally a reversible inhibition of the beating of the cilia of *Vorticella* was also noted. Relatively many of these ciliated protozoa were found attached to the *Nitella* cells by their stalks. In solutions containing 10 µg/ml of cytochalasin B the beating of the cilia stopped, but it went back to normal when the drug was removed from the solution. Stalk contractions were also arrested but did not reappear on removal from the solution. This effect of cytochalasin B to my knowledge has not been reported before.

There are several ways of construing the lack of effect of cytochalasin B on gliding and twitching motility of bacteria: 1) the drug does not penetrate the bacterial cell wall and/or membrane, 2) neither of the two motility phenomena depends on contractile microfilament systems, or 3) procaryotic cells react differently from eucaryotic ones—and obviously different combinations of these possibilities may also be at work. At present it furthermore appears to be a moot question whether cytochalasin B has a direct influence at all on contractile microfilament machinery of any cell type, procaryotic or eucaryotic, or whether it rather acts primarily on other cell elements. *Estensen et al.* (3) have criticized the work of *Wessells et al.* (10) and declared that "the relation between cytochalasin B action, microfilaments and contractile processes is (therefore) by no means well understood". *Sanger & Holtzer* (8) recently reported that cytochalasin B affects cell morphology, cell adhesion and mucopolysaccharide synthesis but has no effect on contractions of isolated skeletal, cardiac and smooth muscle cells and also does not interfere with the contractions of embryonic muscle cells; these authors consider it unlikely that the drug has a direct effect on these kinds of contractile systems. *In vitro* experiments performed by *Forer et al.* (4) showed no action of cytochalasin B on actin, neither on the polymerization of G-actin into F-actin, nor on the binding and subsequent release of heavy meromyosin by actin. Not having any positive controls, *Forer et al.* actually registered no effect of cytochalasin B whatever and consequently might have been working with a totally ineffective preparation of the drug.

Therefore, it does not seem possible for the time being to decide on the correct explanation of the lack of effect of cytochalasin B on gliding and twitching motility of bacteria.

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CELL ADHESION DURING THE CELL LIFE CYCLE

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The initial adhesion of cells in synchronized and steady state cell cultures has been investigated and was found to be of a constant value through all stages of the cell cycle. This is contrary to observations on established cell adhesion in monolayer cell cultures where adhesion is decreased during mitosis. It is suggested that this decrease in cell adhesion is caused by a reduced contact area between the mitotic cell and the substratum.

Observations of monolayer cell cultures show that cells which normally grow in an outstretched and flattened state, usually round up prior to and during mitosis. Such rounded up cells adhere less firmly to the substratum (*Axelrad & McCulloch* 1958, *Terasima & Tolmach* 1963). The mechanism leading to this attenuated binding is not known. It could either be brought about by the reduction of surface area in contact with the substratum when the cell rounds up, or it could stem from a change in the strength of the adhesive forces based on physical changes of the cell surface.

When the cell adhesion is measured in a system which evaluates the initial adhesion of suspended cells to a substratum (*Attramadal & Jonsen* 1970) all contact areas will be of comparable size, as suspended cells are very nearly spherical. This method thus permits a comparison of the adhesiveness of cells which normally have differing contact areas against the substratum. The aim of the present study was to investigate the initial cell adhesion in relation to the cell life cycle.

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MATERIALS AND METHODS

Cell strain and medium. A strain of human skin epithelium cells (ACTC 2544) was used. The cells were propagated in Eagle's minimum essential medium (Grand Island Biological Company, New York, U.S.A.) containing 2.2 g $NaHCO_3$ per litre medium. Per 100 ml medium was added 10 000 I.U. penicillin, 10 mg streptomycin (Glaxo laboratories, Greenford, England) and 10 ml calf serum (National Institute of Public Health, Oslo, Norway). The cells were grown in Roux bottles or Carrel flasks and were detached from these by rolling glass beads gently over the cell layer.

Determination of mitotic activity. The cells were suspended in hypotonic sodium citrate solution (36 mM) for 10 minutes at 37°, and then washed twice in a solution of glacial acetic acid absolute ethanol—1:3. Finally, the cells were suspended in 1 ml of this solution, a few drops of the cell suspension mounted on a slide and dried. The preparations were stained with 2 per cent natural orcein (Gurr, London, England) in 45 per cent acetic acid. The percentage of mitoses was determined by counting at least 2000 cells on each slide.

Synchronization of cell cultures. Synchronizing techniques using cold shocks (*Newton & Wildy* 1959), or the selection of mitotic cells by rinsing monolayer cultures with medium (*Terasima & Tolmach* 1963) gave unreproducible results. A synchronizing procedure based on the addition of excess thymidine was finally elaborated. Maximum mitotic activity was obtained with a single block

of [illegible] mM thymidine in the medium for 22 hours yielding a mitotic activity between 17 5 and 19 1 per cent 11 hours after the end of the thymidine block Incorporation of ^{3}H thymidine was minimal 15 hours after the release of the block Second blocks lasting from 16 to 22 hours, applied at this moment after the first block did not yield a degree of synchrony beyond that obtained from one block The viability of the synchronized cell cultures, assessed by trypan blue uptake was between 98 [illegible] and 99 3 per cent

Cell counting was performed with a Celloscope 101 (AB Lars Ljungberg & Co, Stockholm, Sweden), equipped with a capillary tube of 70 μ internal diameter One ml cell culture was usually diluted with 79 ml 0 15 M sodium chloride solution for counting Each sample was counted in triplicate the mean of which was taken as the count of the sample

Assessment of initial adhesion was performed using glass bead columns (*Attramadal & Jonsen* 1970) The glass bead columns were treated with Hanks balanced salt solution (HBSS) (*Hanks & Wallace* 1949) containing 10 per cent calf serum for 1 hour, and then perfused with 15 ml HBSS prior to use The cells were washed three times in HBSS containing 0 03 M tris buffer pH 7 4 and the adhesion tests were performed in HBSS containing 0 03 M tris buffer

The electrophoretic mobility was measured in a cylindrical chamber according to *Bangham Flemans, Heard & Seaman* (1958) The mobility was determined at 22° in a solution of 0 15 M NaCl containing 10 per cent (v/v) 0 15 M tris buffer pH 7 4 The time needed for a cell to traverse 40 μ in a voltage gradient of 3 25 volts/cm was recorded The current was reversed after each measurement

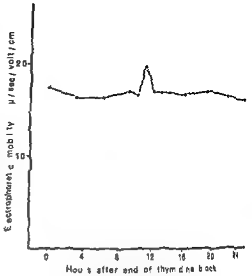

Fig 1 Electrophoretic mobility of NCTC 2544 cells in synchronized cultures

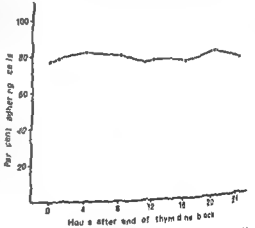

Fig 2 Adhesiveness of suspended NCTC 2544 cells in synchronized cultures

RESULTS

The electrophoretic mobility of NCTC 2544 cells in thymidine synchronized cell cultures was found to be fairly constant through the interphase Fig 1 indicates that an increased mobility occurred 11 hours after releasing the thymidine block This increase was caused by a fraction of the cells having a higher electrophoretic mobility than cells in the interphase At this time the mitotic activity in the culture was 15 20 per cent indicating that the change in surface charge is associated with the mitotic phase

The adhesion of thymidine synchronized cells to glass bead columns was evaluated at various times after the release of the thymidine block but no significant variation of cell adhesion was found through the cell cycle (Fig 2)

To exclude possible interferences from thymidine on the cell adhesion mechanism the adhesion of mitotic cells in normal steady state cell cultures was investigated The mitotic activity in such cultures was 4 1 per cent Samples from steady state cultures which were passed through glass bead columns were found to have a mitotic activity of 4 3 per cent in the suspensions of non attached cells collected from the glass bead

columns In order to evaluate whether these non adhering cells differed from adhering cells non adhering cells from a series of columns were collected After the cell concentration had been adjusted to that of the original cell suspension, samples were again run on glass bead columns and the adhesiveness compared to that of the original cell suspension No significant difference was found 82 9 ± 2 8 per cent of the cells in a normal cell culture adhered to the columns, as compared with 83 0 ± 2 3 per cent of the cells in the non adhering fraction of previous passages of cell suspensions through glass bead columns

DISCUSSION

The observations of *Terasima & Tolmach* (1963) suggest that cell membrane changes which are connected with cell adhesion mechanisms occur prior to and during mitosis *Mayhew* (1966) found a transient increase in the electrophoretic mobility of a human cell line during mitosis and he suggested that this was associated with a higher surface charge from sialic acid at this time in the cell cycle It has been suggested that a direct correlation exists between the magnitude of the negative surface charge and the adhesive properties of cells (*Ambrose & Easty* 1959) The negative charge is in most cells thought mainly to be due to the negative carboxyl groups of N acetyl neuraminic acid (*Cook* 1968) and the removal of sialic acid from the cell surface has been shown to increase the cellular deformability (*Weiss* 1965) *Mitchison & Swann* (1955) found a relatively constant deformability in sea urchin eggs from fertilization until cell division but a rapid increase was observed during cleavage An increased rigidity of the cell surface would enhance the rounding up of a cell and counteract the formation of cell protrusions of small curvature Such protrusions are regarded as an important feature in the development of cell adhesion as they are more able to penetrate the electrostatic repulsion barrier of an approaching surface (*Bangham & Pethica* 1959, *Pethica* 1961) The changes in surface rigidity and surface charge might cause the reduction in cellular adhesion which is observed in monolayer cultures during mitosis and these mechanisms might also counteract initial cell adhesion upon contact

The NCTC 2544 cells were found to have an increased surface charge during mitosis The increase in mobility seems to be restricted to a limited period during the mitotic stage A variation in cell adhesiveness concomitant with the maximum mitotic activity in the synchronized cell cultures was not found Neither were any differences in the initial adhesion between interphase and mitotic cells revealed in steady state cultures, all cells in the cultures were found to possess similar adhesive properties under the experimental conditions used These findings suggest that the initial adhesiveness remains constant during the cell cycle, and that the reduced cell adhesion during mitosis which is observed in other systems (*Axelrad & McCulloc* 1958 *Terasima & Tolmach* 1963) is brought about by a reduced contact area between the cell and the substratum

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VARIATION OF COLONY MORPHOLOGY REFLECTING FIMBRIATION IN *MORAXELLA BOVIS* AND TWO REFERENCE STRAINS OF *M. NONLIQUEFACIENS*

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Five strains of *Moraxella bovis* were studied and found to yield distinct colony types with respect to agar corrosion and formation of corroding spreading zones. The types were largely similar to those of *M. kingii*, although some morphological differences were observed. Distinctly corroding clones, with more or less pronounced spreading colonies (SC type), were isolated from all strains. These isolates were all strongly fimbriated, as observed by electron microscopy. The fimbriae had a diameter of 65–85 Å. Another colony form, without corrosion and spreading (N type), was also observed in all strains. Electron microscopical preparations from these colonies revealed no fimbriae, or only occasional fimbriae, possibly of slightly different appearance, could be detected. With varying frequency, from none observed to about one in 10,000 colonies, N type populations revealed the intracolonial occurrence of fimbriated SC variants by partial change of colony morphology after a few days of incubation. Spontaneous variation from SC to N, accompanied by loss of fimbriation, was also observed. When 12–30 hr old SC colonies were subculuvated, less than one in 10 000 progeny colonies were usually observed to be of the N type, whereas older colonies sometimes revealed a high percentage of N variants in the progeny. A colony type intermediate between N and SC (NSC type) was occasionally seen, with relatively weak agar corrosion and spreading, and with an apparently slightly reduced degree of fimbriation, as compared with the SC type. The intermediate phenotype was unstable, however, showing an irregular tendency to produce progeny of a more corroding and spreading morphology. Two reference strains (including the neotype) of *M. nonliquefaciens* were studied and revealed fimbriae associated colony type differentiation slightly different from previously examined strains of this species and from *M. bovis*.

Spreading-corroding colony forms of *Moraxella kingii* and *M. nonliquefaciens* were described previously (11, 13). The present investigation represents a search for and description of similar colony type variation in *M. bovis*, which is a species closely related to *M. nonliquefaciens* (3, 4). As reported for the latter species (5) and very recently also for *M. kingii* (8), spreading-corroding colonies of those species consist of cells most of which have one or several fimbriae, whereas non-spreading, non-corroding colonies are largely

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without fimbriae The present report also comprises electron microscopical observations of the colony forms of *M bovis*, and an additional examination of two reference strains of *M nonliquefaciens* The latter strains were included as an extension of previous studies (5), since their characteristics appeared to supplement earlier data significantly

MATERIALS AND METHODS

Bacterial Strains

Five strains of *M bovis* were examined the proposed neotype strain, ATCC 10900 (12), and the strains 3, 4, 5 and 9, which were received from Dr A B Pedersen, The Royal Veterinary and Agricultural University, Copenhagen The strain designations were maintained as used by him (14) The species diagnosis of the five strains was based on previous examinations (3, 14), and was confirmed by recent genetic transformation experiments (6) *M nonliquefaciens* 4663/62 (= ATCC 19975 NCTC 10464 proposed as neotype strain (7)) and *M nonliquefaciens* NCTC 7784 have been examined extensively in previous studies (2, 7)

Media and Cultural Conditions

The methods of cultivation for colony type differentiation were the same as those used before (5) with blood agar cultures incubated at 33°C in a very humid atmosphere The blood agar consisted of Tryptose Blood Agar Base (Oxoid) (30 g in 1 litre of distilled water, giving 1 2 per cent agar), to which had been added 0 1 per cent dextrose, 5 per cent human blood (citrated) and NaOH to pH 7 6 Some controls were run with the same agar medium without dextrose and blood and with the original pH of the agar base (7 2) As a rule, 1–4 days old cultures were used for description of the colonies Material for electron microscopial examination was also usually taken from the same kind of blood agar cultures although we also included preparations from younger cultures (down to 5 hr incubation) for supplementary study on some occasions (see Figs and ref 6) For the study of clonal variation, 18 30 hr old colonies were usually spread The exceptions with shorter or longer incubation times before spreading are specified in the Results section Some comparative studies were performed at 37° C Fluid cultures were studied at 33° C in statically incubated 10 mm diameter tubes with 5 ml portions of Mueller Hinton Broth (Difco)

Electron Microscopy of Bacteria Grown on Blood Agar

The agar surface with semiconfluent growth of colonies was scraped with the straight edge of a sterile spatula The material collected was spread by squeezing onto a microscope glass slide and the greyish patches of bacterial mass were allowed to dry slightly

Negative staining was usually done with 0 4 or 1 per cent (w/v) sodium silicotungstate (pH 6 5, but 1 per cent (w/v) potassium phosphotungstate (pH 5 6) and 2 5 per cent (w/v) ammonium molybdate (pH 7) were also used on some occasions

The partially dried material on the slide (see above) was suspended in the negative staining solution by pipetting several times with a narrow orifice pasteur pipette The volume of stain added was sufficient to give a light greyish turbidity One drop of the suspension was put on top of a 400 mesh copper grid covered with a carbon-coated formvar film After 30 sec the excess suspension was removed with a filter paper touching the edge of the grid Often the grids were prepared in a series and examined a few days later with no deleterious effect observed A JEOL JEM 100B microscope was used at 80 kV with a 40 µ objective aperture A primary magnification of 5000 (fixed) was used for scanning and survey pictures ... aken at a mag- ... studied ... y mag- nification Agar controls were made from sterile agar plates incubated in parallel with the cultures and from which specimens were prepared identically These controls did not reveal structures that could be mistaken as fimbriae

Other Methods

See the section of Results

RESULTS

Differentiation of Colony Types

From all five *M bovis* strains clones (cell lines, Table 1) of the non corroding as well as of the corroding colony type were isolated According to the nomenclature introduced by Bovre et al (5) the non spreading and non corroding form was labelled the N type The morphology of such colonies is illustrated in Figs 1 3 and 5 where it is also seen that no corroded area is left on the agar surface after scraping off the bacterial mass The other type of colony labelled SC in the same nomenclature system, grew with depressed

TABLE 1 *Colony Types, Fimbriation and Spontaneous Variation in* Moraxella bovis I

Strain	Colony type II – cell line III	Degree of fimbriation IV	Spontaneous variation of colony morphology: Variant type (cell lines examined) V	Ratio of progeny colonies with partial or complete change VI
ATCC 10900	N a	(—)	SC(SC a SC–b)	$1\ 8\times10^{4}$
	N b	(—)	SC	
	N–c	(—)		
	SC–a	++	N(N b, N–c)	$<1\ 2\times10^{4}$
	SC–b	++	N	
3	N–a	—		$0(<8\ 8\times10^{5})$
	N–b	—	SC(SC–b)	$9\ 3\times10^{5}$
	N–c	—		
	SC–a	++	N(N–c)	$9\ 8\times10$
	SC b	++		
	SC–c	++	N(N a, N–b)	$<2\ 6\times10^{5}$*
			NSC(NSC–a)	$<2\ 6\times10^{5}$*
	NSC a	+	SC	
	N a	—		$0(<7\ 4\times10^{7})$
	N–b	—		$0(<1\ 0\times10^{4})$
	SC–a	++	N(N b)	$<4\ 0\times10^{5}$*
	N a	—		$0(<1\ 0\times10^{4})$
	N–b	—		$0(<9\ 6\times10^{5})$
	N–c	(—)	SC(SC–c)	$<8\ 4\times10^{5}$
	SC–a	++	N(N–a, N–c)	$<9\ 9\times10^{5}$*
	SC–b	++	N	$<7\ 0\times10^{5}$
	SC–c	++		
)	N a	—		$0(<9\ 8\times10^{5})$
	SC–a	++	N	$<8\ 2\times10^{5}$

I The techniques used for colony type differentiation and electron microscopical examination by negative staining are described in the text See also ref 5

II Colony morphology on human blood agar plates (1 2 per cent agar), incubated for 1–4 days at 33° C in a humid atmosphere SC strongly corroding typically with a narrow, corroding spreading zone in the 10900 SC and 5 SC cell lines broad spreading zones, up to 3–4 times the diameter of the colony proper, could be observed N non

variants See Figs 1 9

III The term "cell lines is used for different isolates or clones of each colony type, they have all passed several serial passages (3 to more than 20) of single, typical colonies, but were also often subcultivated *for months* every 1–2 weeks from areas with apparently typical semiconfluent colonies Designations a–c correspond to those used in other studies of these cell lines (6, 9 10)

(\ c

of

sp

several or numerous fimbriae + apparently slightly reduced fimbriation (—) fimbriae occasionally observed in the preparations — no fimbriae detected See Figs 13–19

V Cell lines specified in parentheses are the same as those listed in column 2 Variants with no cell line specification were not studied further Open spaces variants not detected or not examined for (see footnote VI)

VI In N cell lines number of progeny colonies (in relation to total number) with intracolonial sectors larger pits or tonguelike peripheral extensions of SC variant growth In SC lines number of progeny colonies (in relation to total number) with complete change to N (or NSC) type Usually the parent colonies had been spread after 18–30 hr of incubation * Analysis of the progeny from 12–13½ hr old colonies, in a separate set of experiments the progeny from 18–30 hr old colonies of these SC lines revealed N (or 3 NSC) variants at a frequency of about 10^{3} to 10^{4} (see text for experience with older parent colonies) 0 (zero) no SC variants detected even after growth in broth, statically incubated for 2–4 days Open spaces ratios not estimated

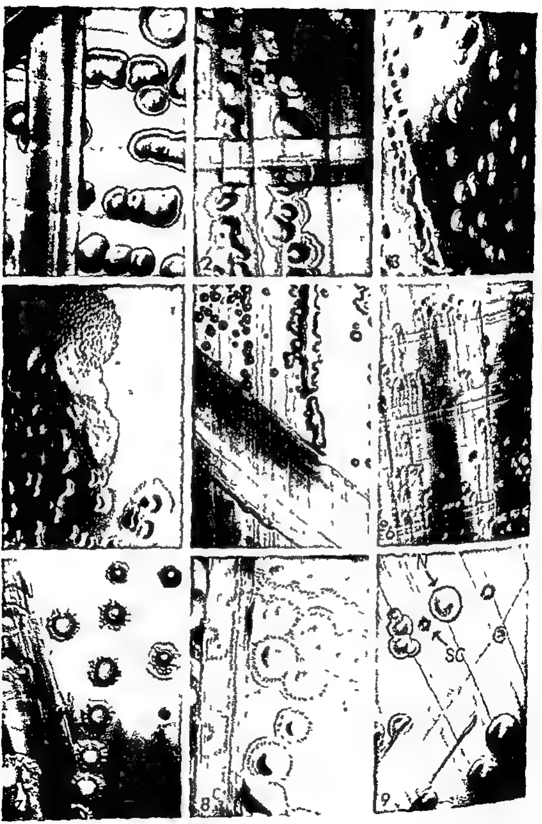

2
3
6
7
8
9
N
SC

Fig 10 *M nonliquefaciens* NCTC 7784 N b, 2 days (7 8 ×)

Fig 11 *M nonliquefaciens* NCTC 7784 SC-a 2 days (7 8 ×)

Fig 12 *M nonliquefaciens* 4663/62, artificial mixture of N-a and SC-a 2 days (5 3 ×)

corroding colonies The depression or corrosion was apparent as rough edged grooves left behind in the medium after scraping The SC colonies had a tendency to form more or less regular spreading zones These zones differed in size from very narrow, almost invisible to the naked eye to rather wide (3–4 times the diameter of the central, raised part of the colony) They usually consisted of a thin layer of growth and were corroding like the rest of the colony The varying morphology of *M bovis* SC colonies and the appearance of the agar surface after scraping off such colonies are shown in Figs 2, 4, 6, 7 and 8 In Fig 9, a mixture of the N and SC forms of strain 9 is shown In this strain, as well as in strain 4, SC colonies were distinctly smaller than the corresponding N colonies, and had only a narrow spreading corroding zone In strain 3 an intermediate type of colony, with almost no spreading and with less corrosion than the ordinary SC form, was sometimes observed It was labelled NSC, as previously used for non spreading and moderately corroding *M nonliquefaciens* variants (5)

Pedersen (14), using different cultural conditions, described two distinct colony types of *M bovis*, one of which was flat and rough and another smaller and smooth type Extensive comparisons showed that the rough form in the terminology of Dr *Pedersen* was corroding when transferred to the conditions used in the present study (*K B Pedersen, L O Froholm & K Bovre*, unpublished) The smooth colony morphology of Dr *Pedersen* almost invariably corresponded to our N type, although we sometimes found that these cell lines formed partly granular suspensions (6)

Fig 1 *M bovis* ATCC 10900 N a blood agar culture incubated for 3 days (4 2 ×)

Fig 2 *M bovis* ATCC 10900 SC-a 3 days (4 2 ×)

Fig 3 *M bovis* 3 N a 3 days (5 4 ×)

Fig 4 *M bovis* 3 SC-a 3 days (5 4 ×)

Fig 5 *M bovis* 5 N a 1 day (7 7 ×)

Fig 6 *M bovis* 5 SC-a 1 day (7 7 ×)

Fig 7 *M bovis* 5 SC-a 2 days (7 6 ×)

Fig 8 *M bovis* 5 SC-b 2 days (4 ×)

Fig 9 *M bovis* 9 mixture of N and SC type colonies 2 days (5 3 ×)

TABLE 2 *Colony Types, Fimbriation and Spontaneous Variation in* Moraxella nonliquefaciens *Reference Strains* I

Strain	Colony type II – cell line III	Degree of fimbriation IV	Spontaneous variation of colony morphology: Variant type (cell lines examined) V	Spontaneous variation of colony morphology: Ratio of progeny colonies with partial or complete change VI
4663/62	N–a	(—)	SC(SC–a)	<1.1×10[illegible]
= ATCC 19975	N–b	(—)		0(<8.6×10[illegible])
= NCTC 10464	SC–a	+ +	N	4.9×10[illegible]
NCTC 7784	N–a	(—)	SC(SC–a)	2.7×10[illegible]
	N–b	(—)	SC(SC–b)	<8.1×10[illegible]
	SC–a	+ +	N	<8.7×10[illegible]
	SC–b	+ +		

I See Table 1, footnote I

II Colony morphology on human blood agar plates (1.2 per cent agar) incubated for 1–4 days at 33° C in a humid atmosphere 4663/62 SC moderately corroding, flat to conical, spreading NCTC 7784 SC strongly to moderately corroding, flat to conical usually spreading N non-corroding conical or hemisphaerical, non spreading See Figs 10–12

III See Table 1, footnote III

IV See Table 1, footnote IV, and Figs 20–23

V VI See Table 1, footnotes V VI Parent colonies spread after 18–30 hr of incubation, see text for experience with older parent colonies

The SC cell lines of the reference *M nonliquefaciens* strains (Table 2) deviated somewhat from those of other strains of this species (5, 13) in being less corroding Although corrosion was still expressed, the most conspicuous character of these SC colonies was their flat, almost transparent appearance after 1 day of incubation as opposed to the conical to hemisphaerical, more opaque N colony type The morphology of 2 days old *M nonliquefaciens* colonies is shown in Figs 10–12 The SC colonies of strain 4663/62 are distinctly more voluminous than the corresponding N type colonies (Fig 12)

The distinctive characteristics of the colony types were almost identical on the blood agar medium described and on the medium without blood The colony characteristics were also essentially similar at 33° C and 37° C, when the humidity was kept high

No fixed correlation was found between the SC character and colony consistency As observed by light microscopy of Gram stained smears and wet preparations, the appearance of the rods and the length of chains they formed, varied independently of N and SC colony type No motility was detected in the wet preparations

The macroscopical appearance of *M bovis* and *M nonliquefaciens* N and SC(NSC) colonies was accompanied by distinct differences seen by agar plate microscopy The N colonies revealed a "compact" periphery and the SC (and to a lesser extent NSC) colonies were surrounded by scattered peripheral cells that expressed the type of translocation called "twitching motility" (10)

In statically incubated *Mueller Hinton* Broth (Difco), the *M bovis* SC cell lines formed an opaque rim sticking to the glass wall close to the surface of the medium after 1–3 days of incubation This "pellicle" was unstable tending to be detached and settle at the bottom when the tube was shaken or on prolonged incubation *In the NSC type*, only traces of a similar surface "pellicle" could occasionally be detected The N type usually showed no such phenomenon When a surface rim was formed from an N cell line this coincided with variation to the SC type (see below) The *M nonliquefaciens* N and SC cell lines examined behaved like those of

M bovis with respect to adherent surface growth in fluid medium although the SC type of strain 7784 developed a distinct surface pellicle comparable to that previously reported for SC isolates of this species (5)

In a limited examination by conventional biochemical tests (2), no difference was observed between the types All *M bovis* cell lines examined were haemolytic Genetic transformation showed a high degree of compatibility between the types although the N cell lines were distinctly less transformable than the corresponding corroding ones (6) Gas chromatography, which was employed rather extensively on this material of strains, usually revealed no significant difference in elution pattern between the N and SC type (9)

Spontaneous Colony Type Variation

The spontaneous variations observed in cell lines of the *M bovis* and *M nonliquefaciens* strains are listed in Tables 1 and 2, with an approximate estimation of variant frequency It should be noted that different SC cell lines of one strain sometimes revealed slight morphological dissimilarities

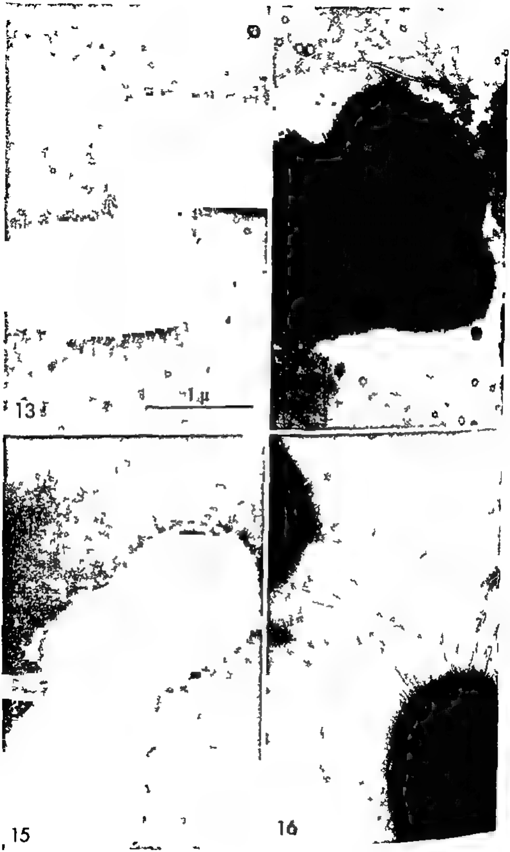
The tabulated results show that N to SC variation is detectable in several *M bovis* and *M nonliquefaciens* strains as indicated previously for the latter species (5) In most cases the occurrence of SC colonies in N populations was extremely infrequent, but the variation could occasionally be observed as pits or sectors of corrosion beneath 2–4 days old colonies or as tonguelike peripheral extensions of corroding growth in both species On some occasions N to SC variation was only detectable when the N cell line in question was incubated statically in broth The formation of a surface rim of growth adherent to the glass wall as described above was indicative of a large proportion SC variant cells present shown by subcultivation from the pellicle

In *M bovis* variation from the SC to N colony type generally appeared to be most pronounced in the early stages after isolation from the parasitic state possibly due to mixed clones These results are not tabulated It was shown however, that some SC cell lines persistently revealed an overwhelming proportion of N variant cells in older colonies, although young colonies could be virtually free of such variants Thus when 2 days old *M bovis* 5 SC–a colonies were spread, sometimes up to 80 per cent N type colonies appeared in the progeny, despite the fact that analysis was undertaken after 15 serial selective passages of the SC colony form When 12–13½ hr old SC colonies were spread however, no N colony variants were detected in the progeny ($<9.9\times10^{-5}$, Table 1) It should be noted that N variants from *M bovis* 10900 SC–a were distinctly enriched also in fluid cultures Often, therefore, the successful maintenance of the *M bovis* SC cell lines was strongly dependent on selective subcultures from young, typical colonies or from spreading corroding outgrowths from older colonies In other cases, however, SC cell lines could be kept apparently pure even by infrequent non selective subcultures on solid medium for several months

The old laboratory strain *M nonliquefaciens* 4663/62 also showed a high proportion of N variants in aged SC colonies In one experiment, a colony of the cell line SC–a of this strain contained less than 10^{-3} N variants after 20 hr of incubation, whereas the same colony had a proportion of more than 50 per cent N colony forming units after 3–4 days of incubation Although the separate N colonies of this strain were smaller than the SC colonies (Fig 12) also these N forms tended to increase in relative number by non selective serial cultures of SC populations on solid medium

Fimbriation of Cells in Relation to Colony Type

Electron microscopy of negatively stained colony material revealed a complete correspondence between the presence of the spreading corroding type of colony and the finding of several or numerous fimbriae at or near the cells (Tables 1 and 2 Figs 13–23) Usually most of the SC type cells appeared fimbriated


13
1 µ
15
16


12
11


1 μ
22

s were situated on a network of fimbriae. This situation is indicated with ++ in the tables. The NSC variant examined appeared only slightly less fimbriated.

Often N type colony material was entirely different, displaying cells with no fimbriae attached or associated (denoted by −). Usually 50–200 cells were closely examined in the microscope each time a preparation was made before this decision was reached. In other cases however, fimbriae were occasionally detected in preparations from N type colonies. This is indicated with (—) in the tables. The fimbriae were in these cases only found infrequently, either as an occasional short filament by itself in the preparation, or as one or at most a few pieces (often very short) attached to a low percentage of cells (Fig. 13). In order to be sure that the finding of free short filaments did not reflect carry over of old fimbriae belonging to old cells in the inoculum several preparations were investigated after a series of single colony isolations. These fimbriae were of the same width as those found associated with distinctly fimbriate SC type cells, but in many cases they seemed somewhat less straight and a little less distinct. This could point to differences between the types of fimbriae. Because the fimbriae could also vary somewhat among the cells in SC type colonies with the age of the colonies and staining conditions, more detailed investigations will be required to resolve the question of different fimbrial types, however.

The fimbriae were often concentrated near the polar regions of the cells and the impression was that numerous fimbriae actually originated from each cell in many cases (Fig. 16). The SC fimbriae were straight and seemed stiff, apparently easily broken during preparation. Often a few fimbriae were oriented in parallel over long stretches. Tight side to side association with distinct bundle formation is evident in Figs. 14, 21 and 23. The width of individual fimbriae was found to be 65–85 Å (see ref. 5 for measurement of width). Slight thickness variation could occasionally be observed.

Fig. 13. M. bovis ATCC 10900 N–a from blood agar culture grown for 1 day, arrow pointing at a single fimbria (25 000 ×).

Fig. 14. M. bovis ATCC 10900 SC–b, 1 day (25 000 ×).

Fig. 15. M. bovis 3 N–a 3 days (95 000 ×).

Fig. 16. M. bovis 3 SC–c 12 hr (25 000 ×).

Fig. 17. M. bovis 5 N–a 12 hr (27 000 ×).

Fig. 18. M. bovis 5 SC–a 16 hr (27,000 ×).

Fig. 19. M. bovis 5 SC–b 12 hr (27 000 ×).

Fig. 20. M. nonliquefaciens NCTC 7784 N–b, 1 day (27,000 ×).

Fig. 21. M. nonliquefaciens NCTC 7784 streptomycin resistant transformant with SC colony morphology arising from N–b (see ref. 6), 10 hr (25 000 ×).

Fig. 22. M. nonliquefaciens 4663/62 N–a 1 day (27 000 ×).

Fig. 23. M. nonliquefaciens 4663/62 SC–a 7 hr (27 000 ×).

DISCUSSION

The finding of association between the spreading corroding colony type and fimbriation previously reported for *M. nonliquefaciens* (5) has been confirmed by the additional study of two reference strains of this species. It has also been proved that the closely related species *M. bovis* exhibits similar colony type differentiation, and the association between colony characteristics and fimbriation appears analogous in this organism. Corresponding findings in the distantly related *M. kingii* are reported separately (8).

In the data presented there is no exception to the rule that strong fimbriation is correlated with the spreading corroding colony characteristics, although the latter may vary somewhat in their expression. However, it is important to note that the non spreading non corroding colony morphology can be associated both with absence of fimbriae and with the occurrence of a few fimbriae in the preparations.

Intertype covariation of colony morphology and fimbriation has been shown to occur spontaneously in either direction. However, estimation of variation frequency is strongly

complicated by differential behaviour of the fimbriated (i e, SC) and non-fimbriated (N) variants when they occur simultaneously within a colony, as well as in broth culture. Thus, the SC forms with strong spreading tendency may become predominant in a large part of the N colony where it arises. On the other hand, N variants within an SC colony are often enriched as the colony ages, and within relatively short time such a colony may contain more living cells of the variant than of the original type, for reasons that are not known in detail. Under these conditions, the usual screening of progeny from single colonies for such variants may possibly lead to an overestimation of genetic variation frequency, especially from SC to N. Therefore, the presented data on spontaneous emergence of variants may be compatible with a low-frequent variational mechanism in most or all cases. It should be noted that apparently stable N variants, both with and without occasional fimbriae, do exist. However, extensive search may show that they are not completely stable, since the variation from N to SC is often only irregularly detectable. For comparison, it should be mentioned that the change from type-1 fimbriation to non-fimbriation in *Escherichia coli* is described as an all-or-nothing sudden, random, spontaneous most often reversible event, the frequency of which is strongly affected by the environment (1).

The genetic mechanism of fimbrial variation in moraxellae is unknown. *Brinton* (1) states that type-1 fimbriae of *E coli* are controlled by a structural gene or genes located on the chromosome, and also by regulator genes which have not yet been mapped, whereas the sex fimbriae appear to be controlled by the episomes they transfer.

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FIMBRIATION ASSOCIATED WITH THE SPREADING-CORRODING COLONY TYPE IN *MORAXELLA KINGII*

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In four out of five *Moraxella kingii* strains examined, strongly corroding and usually spreading colony forms (named SC type) could be isolated, as well as non spreading and non corroding or weakly corroding forms (N colony type) Electron microscopical preparations made from SC colonies always contained numerous fimbriae, whereas usually no fimbriae could be detected in preparations made from cultures of the N type In the case of weakly corroding forms, a few fimbriae could be observed in some preparations, their occurrence apparently corresponding in time with the slowly developing corrosion The fimbriae of *M kingii* were of the same approximate dimensions (60–85 Å) as observed for spreading corroding colony variants of *M nonliquefaciens* and *M bovis* In one strain, low frequent spontaneous variation from the N to SC type was observed, with a corresponding gain of fimbriation Spontaneous variation in the other direction, from SC to N, accompanied by loss of fimbriation was also seen in this strain The latter variation was probably also always of a low frequency When it had occurred the N variant cells easily outnumbered the SC type in mixed culture

In 1969, *Henriksen* (8) described a colony type of *Moraxella kingii* that corroded agar and showed considerable peripheral spread, contrary to the ordinary smooth colony type In the same year, *Henriksen & Bovre* (10) reported similar colony types in *M nonliquefaciens* The finding by *Bovre et al* (2) that the spreading corroding character of the latter species is associated with the occurrence of strongly fimbriated cells, stimulated the search for a similar correlation in other bacterial species In the preceding paper, fimbriae associated colony type variation was described in *M bovis* (3) The present report concerns an extended investigation on colony type variation in *M kingii*, with electron microscopy of the variants

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MATERIALS AND METHODS

Bacterial Strains

The strains of *M kingii* examined were 4177/66 (= ATCC 23330, NCTC 10529, proposed as type strain (9)), CDC A1702 and CDC 5530 (both received from the late Miss *Elizabeth O King*, Communicable Disease Center Atlanta, Georgia, USA), 8994/70 and 9076/70 The two latter strains and the first one were isolated from clinical specimens at Rikshospitalet, Oslo, Norway The strains 4177/66 A1702 and 5530 have been examined extensively in a previous investigation, where their characteristics and intraspecies affinities have been established by conventional and genetic means (9) The two remaining strains were

identified as *M kingii* by some of these methods including genetic transformation

Methods for the Study of Colony Type and Fimbriation

The media and procedures of cultivation for type differentiation were the same as those used previously (3) Electron microscopy of material collected from agar surface growth was performed as described (3) Some other techniques are referred to in the section of Results The respective cell lines (see Table 1, footnote III) were kept in culture for months by inoculation on blood agar plates from typical separate or semiconfluent colonies every fourth to fifth day

RESULTS

Differentiation of Colony Types

In all strains except 5530, both non corroding and distinctly corroding colony forms were observed and labelled N (non-spreading and non-corroding), resp SC (spreading corroding) type according to the nomenclature introduced for corresponding types in other species (2, 3) The cell lines examined are listed in Table 1

The colony morphology of representative cell lines of the two most thoroughly examined strains is shown in Figs 1 5, with the demonstration by scraping with a platinum loop of corroded areas on the agar surface only corresponding to SC colonies Although colony forms labelled N were usually completely non corroding, even after several days of incubation, there were two exceptions (Table 1) In cases where a colony of 4177/66 N–a grew well isolated and therefore became very large, a weak corrosion could be detected centrally beneath the colony after 2–4 days of incubation (not apparent in Fig 1) The non spreading 5530 (N)–b also showed a slow development of weak corrosion usually not apparent by scraping before approximately one day of incubation Contrary to this all cell lines labelled SC revealed distinct corrosion at the first sign of growth, before raised colonies could be detected with a hand lens In many cases not illustrated, the peripheral spread of 4177/66 A1702 and 9076/70 SC type colonies was more pronounced than shown in Figs 2, 4 and 5, as also found by *Henriksen* in other strains of *M kingii* (8) On the other hand, the SC type of 8994/70 studied revealed no macroscopically visible spreading zone The characteristics of N and SC cell lines were distinct and analogous on agar media with and without blood (agar medium without blood was only exceptionally used and there was no major change when the incubation temperature was increased from the usual 33° C to 37° C

SC type colonies had a slightly "dry" consistency This characteristic was only occasionally shared by N type colonies There was no correlation between cell shape size and tendency to form chains and the type of colony formed No motility could be detected by ordinary light microscopy of wet preparations By agar plate microscopy of 4177/66 A1702 and 9076/70 colony variants N type colonies appeared sharply circumscribed whereas SC colonies were surrounded by scattered cells or pairs of cells showing movement in small "jumps", characteristical of "twitching motility This part of the studies has been reported separately, together with similar observations in *M nonliquefaciens* and *M bovis* (7)

Like the SC colony types of *M nonliquefaciens* and *M bovis*, also the *M kingii SC* type often formed a surface pellicle or a rim of growth sticking to the glass tube wall a little below the surface of Mueller Hinton Broth (Difco) within 1–2 days of static incubation contrary to the *N* type Although observable with all SC cell lines tested the phenomenon appeared less regular than in other species of *Moraxella* (2 4)

There was no demonstrable difference between corresponding *N* and *SC* forms when examined by some conventional diagnostic tests (9) except for the characteristics mentioned This search for biochemical differences was not extensive

In genetic transformation experiments the compatibility was high between N type donors of transforming *DNA* and SC type recipient cells The *N* forms were distinctly less

TABLE I *Colony Types, Fimbriation and Spontaneous Variation in* Moraxella kingii [I]

Strain	Colony type [II] – cell line [III]	Degree of fimbriation [IV]	Spontaneous variation of colony morphology: Variant type (cell lines examined) [V]	Spontaneous variation of colony morphology: Ratio of progeny colonies with partial or complete change [VI]
4177/66	N–a	(—)		$<7.8\times10^{-5}$*
= ATCC 23330	N–b	—		
= NCTG 10529	SC–a	+ +	N-like, reverting	8.9×10^{-5}
CDC A1702	N–a	—		$<5.1\times10^{-5}$*
	N–b	—		
	N–c	—	SC(SC–c)	$<4.8\times10^{-5}$
	N–d	—	SC(SC–d)	$<8.3\times10^{-5}$
	N–e	—		$<2.0\times10^{-5}$*
	N–f	—		
	SC–a	+ +	N(N–b, N–c, N–d, N–e)	$<4.5\times10^{-5}$
	SC–b	+ +	N–f	$<8.5\times10^{-5}$
	SC–c	+ +	N	
	SC–d	+ +		
CDC 5530	N–a	—		
	(N)–b	(—+)		
8994/70	N–a	—		$<6.1\times10^{-5}$*
	SC–a	+	N	$<8.9\times10^{-5}$
9076/70	N–a	—		$<6.9\times10^{-5}$*
	SC–a	+ +	N-like, reverting	5.7×10^{-6}

[I] The techniques used for colony type differentiation and electron microscopical examination by negative staining were, with few modifications, as described by *Bøvre & Frøholm* (3).

[II] Colony morphology on human blood agar plates (1.2 per cent agar) incubated for 2–4 days at 33° C in a humid atmosphere. SC: strongly corroding, typically with a wide, corroding spreading zone, up to 3–4 times the diameter of the colony proper. 8994/70 SC had no macroscopically visible spreading zone. N: non-spreading, generally non-corroding. 4177/66 N–a exhibited a weak, central corrosion beneath very large, isolated colonies after 2 days of incubation; in CDC 5530 (N)–b there was a slow development of weak corrosion, apparent after 1 day. See Figs. 1–5.

[III] The term "cell lines" is used for different isolates or clones of each colony type; they have all passed several serial passages (3 to more than 20) of single, typical colonies, but were also often subcultivated less selectively for long periods (see Materials and Methods). Cell line designations (a–f) correspond to those used in other studies of these variants (4, 7).

[IV] Semiquantitative estimates by electron microscopy of 5 hr to 4 days old cultures (cultural conditions as specified in footnote II). + +: most cells with several or numerous fimbriae. +: apparently slightly reduced fimbriation. (—+): a moderate number of fimbriae observed in some preparations, preferentially from old cultures. (—): fimbriae only occasionally observed, preferentially from old cultures. —: no fimbriae detected. See Figs. 6–10.

[V] Cell lines specified in parentheses appear again in column 2. N-like, reverting: an apparently temporary phenotypic change; later subculture revealed typical SC progeny. Open spaces: variants not detected or not examined for (see footnote VI).

[VI] In N cell lines: number of progeny colonies (in relation to total number) showing any partial change indicating the intracolonial existence of genetically stable variants with increased corroding (or spreading) capacity. In SC lines: number of progeny colonies (in relation to total number) with complete change to the N type; age of parent colonies 1–2 days. * No SC variants detected even after growth in Mueller Hinton Broth (Difco), statically incubated for 2–4 days. Open spaces: ratios not estimated.

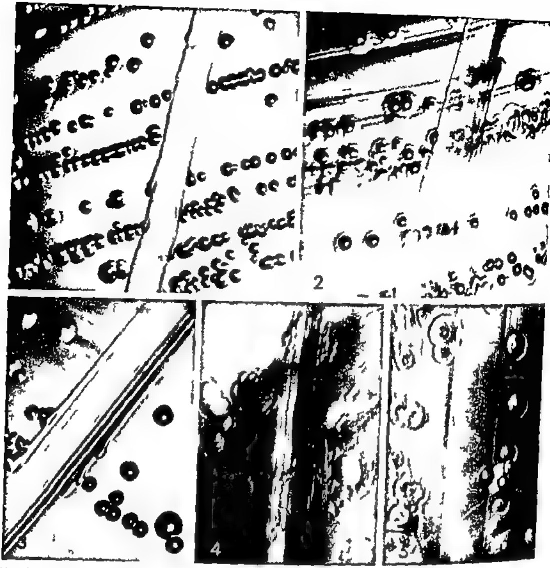

Fig 1 *M k ng* i 4177/66 N a blood agar culture incubated for 3 days (4 5 ×)

Fig 2 4177/66 SC–a 3 days (4 5 ×)

Fig 3 A1702 N–a 3 days (4 5 ×)

Fig 4 A1702 SC–a 3 days (4 5 ×)

Fig 5 A1702 SC a 1 day two N type colonies seen peripherally n a large ra sed SC type co ony in lower right quadrant (7 6 ×)

transformable however (4) Gas chromato graphy of 4177/66 and 9076/70 N and SC types revealed essentially identical elution profiles (6) These overall affinities are con sistent with the clonal intertype variations described below

Spontaneous Colony Type Variation

Approximate estimates (usually upper lim its) of colony type variant freq ency are given in Table 1 for most N and SC cell lines In four of the strains the cell lines studied all apparently originated as segregants from mixed populations in the early stages of the study Except for not further exam ned N colony variants from 8994/70 SC–a and a

seemingly transient phenotypic variation of low frequency from SC to 'N' in two other strains, the cloned cell lines of these four strains seemed completely stable In the fifth strain (A1702), bidirectional clonal variation was observed between the two colony types Also in this strain, however, any variation was probably of low frequency, comparable to most cases of colony type variation in *M bovis* and *M nonliquefaciens* N and SC cell lines (3)

The spontaneous variation from A1702 N to SC colony type was observed only a couple of times as spreading corroding tongues extending from the periphery of confluent growth areas during months of subcultivation of the various N cell lines The variation was not observed within the altogether 10 well isolated colonies examined in the assay of variant frequency (Table 1) No SC variants could be isolated from statically incubated Mueller Hinton Broth (Difco) cultures of the N forms of this or any other *M kingii* strain when attempted

Spontaneous variation from SC to N type in strain A1702 appeared to be more frequent than variation in the opposite direction When SC type populations of this strain were serially transferred on solid medium every fourth to fifth day, the proportion of progeny colonies with N characteristics was sometimes as high as 1–10 per cent probably due to preformed variants, not detected in the inoculum, and possibly with slightly higher survival capacity than the SC type N type cell enrichment was sometimes observed also in the variant 8994/70 SC-a cell line under similar conditions When single SC colonies were spread after 1–2 days in the actual analysis of variation, however, no N variants were detected (Table 1) As also observed in a strain of *M bovis* (3) prolonged incubation in Mueller Hinton Broth of some *M kingii* SC cell lines resulted in significant enrichment of N variant forms Thus, a broth culture of A1702 SC-a with about 0 01 per cent N variant cells at 19 hr of incubation gradually changed to a proportion of close to 100 per cent N cells at 7 days A duplicate culture had become sterile at this time, showing that 7 days were close to the maximum survival time

The A1702 N-b and N-f variants originated from the respective SC parent lines (Table 1) by isolation from fluid cultures. However, experiments in fluid medium with SC cultures of the other strains of *M kingii* did not result in detection of N variants

It should be stressed that the tendency of N variants to appear and increase strongly in relative proportion in SC type populations, as particularly looked for during months of serial passages on solid medium, was observed in only two strains Even in these strains the variation was inapparent for long periods Therefore, it is possible that the stability of these SC cell lines changed during the investigation

Electron Microscopy

In a high resolution electron microscope fimbriae are generally easy to recognize in negatively stained preparations as thin threads of even diameter and straight appearance for extended lengths When few and short fimbriae are present, they might be difficult to see, especially when the stain accumulates near the cells Short fimbriae can, obviously, also hide under or above the cells Because of this it was necessary to scrutinize many cells (50–100) and also to take a number of micrographs for subsequent examination in case of negative findings In some cases (see below) it was found important to include examination of young cultures (5 24 hr of growth) to clearly see the difference in fimbriation between the cell types, and it was also in these cases important to inoculate from young cultures, to avoid bringing fimbriae in with the inoculum The fimbriae are mostly very long structures and several may originate in one cell Hence, fimbriae from one variant cell may "contaminate a preparation with many otherwise completely unfimbriated cells

The results of electron microscopical examinations of the various *M kingii* cell lines are given in Table 1 As can be seen, there is

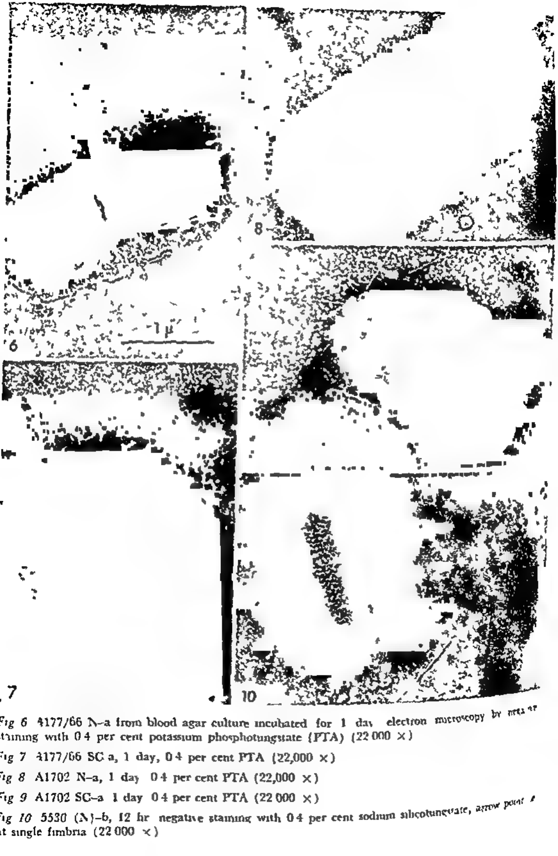

Fig 6 4177/66 N–a from blood agar culture incubated for 1 day electron microscopy by negative staining with 0·4 per cent potassium phosphotungstate (PTA) (22 000 ×)

Fig 7 4177/66 SC–a, 1 day, 0·4 per cent PTA (22,000 ×)

Fig 8 A1702 N–a, 1 day 0·4 per cent PTA (22,000 ×)

Fig 9 A1702 SC–a 1 day 0·4 per cent PTA (22 000 ×)

Fig 10 5530 (N)–b, 12 hr negative staining with 0·4 per cent sodium silicotungstate, arrow points at single fimbria (22 000 ×)

a general correlation between the spreading-corroding colony morphology and the occurrence of fimbriated cells in the preparations The observations seemed reproducible within each cell line when it was investigated repeatedly under similar conditions Preparations of the two N cell lines with slowly developing corrosion (4177/66 N-a and 5530 (N)-b) sometimes revealed a single or a few fimbriae, but not before 12–24 hr of growth The SC cell lines were always fimbriated, at early and late stages of growth. Fig 6 shows a typical diplobacillus of *M kingii* 4177/66 N-a There are no fimbriae, but a number of other structures can be seen extracellularly Most of this is in the form of granular material but also a few vesicle- or bleblike structures appear all around the cells Above the cleavage furrow a structure possibly representing wall material from a disintegrated cell, is evident Fig 7 is an example of a *M kingii* 4177/66 SC-a cell with two bundles of fimbriae Large masses of fimbriae were occasionally seen in this cell line It is impossible to say if any of the fimbriae shown originate in the cell illustrated Fig 8 demonstrates that A1702 N-a is without fimbriae and also in other respects resembles 4177/66 N-a (Fig 6). In Fig 9 are shown two cells of A1702 SC-a together with many fimbriae, some single and some in more or less tight bundles Fig 10 is of a single cell of 5530 (N)-b with one short fimbria originating close to one of the cell poles as indicated by an arrow

It should be stressed here that quantitative enumerations are difficult with negative stain preparations As indicated, it is also often difficult to assign the fimbriae detected to one particular cell in a preparation Methods for true estimation of number of fimbriae per cell origin on the cell surface and length of individual fimbriae are difficult to devise The fimbriae are often found loose in the preparations hence fragmentation must also commonly take place Because of all these difficulties we have refrained from a detailed study of quantitative aspects of the fimbriation for the time being However for comparison with other studies of fimbriae in bacteria a few approximate dimensions and numbers are given in the following We hope to supplement these with more accurate data in the future

The width of *M kingii* fimbriae was estimated to be in the range 60–85 Å by measuring center-to-center distance where fimbriae were running close in parallel (see ref 2 for discussion of measurements and further references) Lengths were variable from short pieces to several μ The fimbriae were regularly long when appearing in large masses and occasionally when they were few The number was variable from one detected to probably many hundreds per bacterium The fimbriae tended to orient in parallel, and in a few SC cell lines many fimbriae were always found closely packed in bundles When many fimbriae were seen, they were often distributed all over the cells When they were few, they most often originated in some rather typical locations (*e g*, subpolar or in the cleavage furrow between cells)

No fimbrial substructure that was clearly different from the phase contrast granularity of the electron micrographs has been detected *in this stage of the work* Occasionally, there was some thickness variation along the fimbriae that could be explained by the presence of some helical structure with a long period

DISCUSSION

As previously found for the closely interrelated species *M nonliquefaciens* and *M bovis* (2, 3) the distantly related *M kingii* (9) reveals the occurrence of an agar-corroding (and usually spreading) SC colony type combined with a large proportion of richly fimbriated cells in electron microscopical preparations As in the other species, a non-corroding or weakly corroding N colony type is also found which at least when studied early in growth shows very few fimbriae or, usually none at all

Bidirectional variation between the two main colony/fimbriation types has been ob-

served in only one out of five strains (A1702). The detection rate of N to SC variation was low even in this strain, indicating a somewhat greater genetic stability of *M. kingii* N forms than of those in the other species. However, the more easily detectable A1702 SC to N variation showed that the latter type also in this species is inadvertently enriched under laboratory conditions, as previously shown for *M. nonliquefaciens* and *M. bovis* (3). In two other strains, only transient N types were found occasionally in the SC populations. An N type cell line of one of these strains (4177/66 N–a, Table 1) showed late appearing corrosion corresponding to late appearance of fimbriae. No stable SC variant was detected from such colonies, however. These facts could indicate the existence also of phenotypic changes, possibly due to repression-derepression occurring intracolonially.

Firm quantitative data about fimbriae as well as structural analysis of them have not been considered part of the present study. Generally, the fimbriae of *M. kingii* are similar to those of *M. nonliquefaciens* (3, 4) and *M. bovis* (4) in occurrence and dimensions. The slightly different ranges of width are not considered significant at the moment. The possibility of several different kinds of fimbriae occurring together is still open. Some of the fimbriae found in N type colonies, especially the short ones, could be of a type different from the majority of those found attached to SC cells. No systematic test for mannose sensitive adhesin has been performed so we cannot say whether the fimbriae of moraxellae are all alike in this respect and identical to some of the fimbrial types proposed by *Duguid* (5) or *Brinton* (1).

Because of the close correlation detected between fimbriation and colony type it is tempting to believe that certain aspects of colony morphology and special cultural behaviour (*e. g.*, agar corrosion and pellicle formation) may be explained by the chemical and physical properties of fimbriae. This and the elucidation of the possible participation of fimbriae in "twitching motility" (7) and genetic transformation (4), must await future investigations.

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COMPETENCE IN GENETIC TRANSFORMATION RELATED TO COLONY TYPE AND FIMBRIATION IN THREE SPECIES OF *MORAXELLA*

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Non-fimbriated or weakly fimbriated cells from non-corroding colonies (N type) and strongly fimbriated cells from agar-corroding and often spreading colonies (SC or NSC types) of the bacterial species *Moraxella nonliquefaciens*, *M. bovis* and *M. kingii* were found to differ very distinctly when used as recipients in streptomycin resistance transformation. N type isolates of these species were always found to be deficient in competence, being either non-transformable or weakly responding when exposed to autologous or homologous DNA extracted from streptomycin resistant mutants of N cells. Corresponding SC or NSC type isolates revealed much higher T/E (transformants/colony forming recipient units) ratios when exposed to the same N type DNA preparations ($T/E = 10^{-3}$ to 10^{-2} or above in 11 strains, 10^{-4} to 10^{-5} in three strains: short term (20 min) DNA exposure). These values are from about 400 times to more than 10^{4} times higher than the N type T/E ratios in individual strains. In the three strains with low SC or NSC T/E ratios the corresponding N forms were either transformable by long term DNA exposure only or completely non-transformable by any method. By using a modification of the long term DNA exposure technique for screening of competence on more than 14,000 single colonies, no spontaneous change of competence independent of variation in colony type and fimbriation could be detected in any direction, except for one single observation of genetically stable change from low level competence to incompetence in an N type variant. It is briefly discussed whether fimbriae could play a hitherto unknown active role as an important competence factor or whether the cellular appendages may be functionally inert in this respect, only reflecting the presence of one or more such factor(s).

In *Moraxella nonliquefaciens*, *M. bovis* and *M. kingii*, variation between colony types is associated with difference in fimbriation of component cells, as studied by electron microscopy (5, 8, 9), and also with changed ability of the cells to perform the kind of surface translocation named "twitching motility", as studied by agar plate microscopy (11). Thus, one group of variant substrains, labelled SC and NSC types, form agar-corroding colonies, usually surrounded by spreading-corroding surface zones of growth, and consist of cells that are strongly fimbriated. The spreading zones of these colonies are composed of "twitching" cells. The other main form (N type) has non-corroding, non-spreading colonies, consisting of cells that are largely non-fimbriated and without ability to "twitch".

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served in only one out of five strains (A1702). The detection rate of N to SC variation was low even in this strain, indicating a somewhat greater genetic stability of *M. kingii* N forms than of those in the other species. However, the more easily detectable A1702 SC to N variation showed that the latter type also in this species is inadvertently enriched under laboratory conditions, as previously shown for *M. nonliquefaciens* and *M. bovis* (3). In two other strains, only transient N types were found occasionally in the SC populations. An N type cell line of one of these strains (4177/66 N-a, Table 1) showed late appearing corrosion corresponding to late appearance of fimbriae. No stable SC variant was detected from such colonies, however. These facts could indicate the existence also of phenotypic changes, possibly due to repression-derepression occurring intracolonially.

Firm quantitative data about fimbriae as well as structural analysis of them have not been considered part of the present study. Generally, the fimbriae of *M. kingii* are similar to those of *M. nonliquefaciens* (3, 4) and *M. bovis* (4) in occurrence and dimensions. The slightly different ranges of width are not considered significant at the moment. The possibility of several different kinds of fimbriae occurring together is still open. Some of the fimbriae found in N type colonies, especially the short ones, could be of a type different from the majority of those found attached to SC cells. No systematic test for mannose sensitive adhesin has been performed so we cannot say whether the fimbriae of moraxellae are all alike in this respect and identical to some of the fimbrial types proposed by *Duguid* (5) or *Brinton* (1).

Because of the close correlation detected between fimbriation and colony type it is tempting to believe that certain aspects of colony morphology and special cultural behaviour (*e.g.*, agar corrosion and pellicle formation) may be explained by the chemical and physical properties of fimbriae. This and the elucidation of the possible participation of fimbriae in "twitching motility" (7) and genetic transformation (4), must await future investigations.

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forms grew quite fast. Thus non transformed SC clones occasionally occurred, observed to be high transformable in repeated tests. Therefore, the method was used in the following manner: a large inoculum (about half a 1–2 days old colony) was carefully spread in an area of about 1 cm², and streptomycin (50 µg/ml) was added at a stage of considerable growth in the whole area (5½ to 6½ hr or more). Under these conditions a rather high number of transformants was seen even in some cell lines with T/E ratios below 10^{-4} (procedure a)), and these transformant counts were occasionally difficult to distinguish clearly from the confluent growth of transformants seen in cell lines with T/E ratios around 10^{-2}. For these reasons Jyssum's method was mainly used for distinction between incompetence on the one hand and competence on the other, and not for analysis of variation between the numerous possible levels of competence (Tables 4 and 5). However, as presented in Table 6, if the yield of transformants was reduced by using heterologous but related donor DNA (as *M. bovis* DNA towards *M. nonliquefaciens* recipients), the method was applicable for studying variation from high level to low level competence, or *vice versa*.

Other Methods

Electron microscopical technique and other procedures used to supplement previous results have been described (8) or are presented in the Results section.

TABLE 1. *Relation between Fimbriation and Competence of Genetic Transformation in* Moraxella nonliquefaciens I

Strain	Colony type II cell line III	Degree of fimbriation IV	Competence (T/E ratio) V
4663/62	N a	(—)	$3\,9 \times 10^{-4}$
= ATCC 19975	N–b	(—)*	$0(<1 \times 10^{-8})$
= NCTC 10464	SC–a	+ +	$2\,5 \times 10^{-2}$
NCTC 7784	N a	(—)	$1\,7 \times 10^{-8}$
	N b	(—)*	$8\,2 \times 10^{-7}$
	SC–a	+ +	$1\,8 \times 10^{-2}$
	SC–b	+ +	$1\,1 \times 10^{-2}$
3067/66	N a	—	$0(<4 \times 10^{-6})$
	SC–a	+ +	$3\,8 \times 10^{-5}$
	NSC–a	+	$3\,8 \times 10^{-6}$
3179/66	N a	—	$1\,3 \times 10^{-8}$
	SC–a	+ +	$5\,3 \times 10^{-3}$
	SC–b	+ +	$6\,8 \times 10^{-3}$
3832/66	N a	—	$<4 \times 10^{-8}$
	SC a	+ +	$8\,1 \times 10^{-3}$

I Quantitative streptomycin resistance transformation was employed as described by *Bøvre* (2) with few modifications. The DNA exposure was terminated after 20 min by means of DNase. The transforming DNA originated from *M. nonliquefaciens*, always as streptomycin resistant mutants of N type cell lines. In the case of 4663/62 and 3067/66 recipients autologous N type DNA was used, i.e. from the same strain.

II SC: spreading, most often distinctly corroding. N: non spreading, non-corroding. NSC: moderately corroding, non spreading. See separate descriptions of colony types (5, 8).

III Cell line designations for the strains 4663/62 and 7784 (labels a, b) correspond to those used in other studies of these variants (8–11). See Materials and Methods for labelling of cell lines of the other strains.

IV + +: most cells with several or numerous fimbriae. +: apparently slightly reduced fimbriation. (—): fimbriae occasionally observed in the preparations. (—)*: fimbriae occasionally observed, but not in young cultures (about 7 hr) as those used as recipients in transformation. —: no fimbriae detected. See separate reports for electron microscopical data (5, 11).

V T = number of streptomycin resistant transformants per ml. E = number of colony forming units/ml exposed to transforming DNA. 0 in this column indicates that no transformants were detected, even when DNase was omitted from the transformation system.

RESULTS

Genetic Competence Related to Colony Type and Fimbriation

The transformability in streptomycin resistance transformation of *M nonliquefaciens* cell lines is shown in Table 1 There is a uniform and striking difference in terms of T/E ratios between N type cell lines (with none or only occasional fimbriae in the electron microscopical preparations) and the corresponding SC (and NSC) cell lines (generally with strongly fimbriated cells) The T/E ratios of the N colony type are all less than about 1/400 (most often 1/1000 or lower) of the T/E ratios of the correspo ding SC (NSC) type In several cases N cell lines are either non transformable in long term DNA exposure or show only infrequent occurrence of transformants However the highest N type T/E ratios of three strains (those with the highest T/E ratios for SC are comparable to the T/E ratios of the most fimbriated cell lines isolated and studied from the two remaining strains. In strain 4663/62 the completely non transformable cell line N b (Table 1) revealed no fimbr... at the early cultural stage (5-7 hr) when

TABLE 2 *Relation between Fimbriation and Competence of Genetic Transformation in Moraxella bovis* I

Strain	Colony type II cell line III	Degree of fimbriation IV	Competence (T/E ratio) V
ATCC 10900	N a	(•	2 3×10
	N b	(	$2 4\times10^{-5}$
	N c	(	$3 6\times10^{-5}$
	SC a		$1 6\times10^{-2}$
	SC b	+	$2 2\times10^{-}$
3	N a	—	$0 <9\times10^{-}$
	N b	—	$8 5\times10^{-}$
	N c	—	$5 0\times10^{-}$
	SC a	+ +	$1 ?\times10^{-}$
	SC b	+	1 2×10
	SC c	+	$8 7\times10^{-}$
	NSC a		$5 8\times10^{-}$
4	N a		$3 6\times10^{-}$
	N b		$2 7\times10^{-}$
	SC a	+	1 9×10
5	N a		$0 (<3\times10^{-})$
	N b		$1 ?\times10^{-}$
	N c		$1 5\times10^{-}$
	SC a	+ +	$8 3\times10^{-}$
	SC b	+	1 8×10
	SC c	+ +	1 0×10
9	N a		$7 1\times10^{-}$
	SC a	+ +	$?7\times10^{-}$

I Quantitative streptomycin resistance transformation as specified in Table 1 footnote I The transforming DNA was extracted from *M bovis* Usually the cell line 10900 N a was used as donor In the case of the two strain 9 recipients *M bovis* 3 N-a was the source of transforming DNA

II SC strongly corroding with corroding spreading zones of different width N non spreading non corroding NSC moderately corroding weakly not spread g See separate descriptions for details in colony type variation (8)

III Cell line designations correspond to those used elsewhere for these variants (8 11)

IV See Table 1 footnote IV and the electron microscopical data (8)

V See Table 1 footnote V

recipients were harvested for transformation, whereas the low competent cell line N–a from which N–b was derived (see legend to Table 5 footnote IV), revealed occasional fimbriae in the preparations also at this stage of growth Similarly, the very low competent cell line 7784 N–b revealed occasional fimbriae in the electron microscopical preparations only at a later stage of growth The corresponding high competent SC cell lines were strongly fimbriated also at the pin point colony stage

In the *M bovis* strains examined (Table 2) the distinctly fimbriated SC and NSC cell lines are, without exception, highly competent (T/E ratios above 10^{-4}), whereas the N colony type is either low competent (maximum T/E ratio 8.5×10^{-5}) or incompetent as proved by long-term DNA exposure The low competent 10900 N–a cell line revealed no fimbriae in preparations made from 7 hr old cultures, but occasionally fimbriae were detected after incubation for 30 hr The SC–b line of the same strain was found to be strongly fimbriated when examined at 7 hr

In *M kingii* (Table 3), all six SC type isolates examined from three of the strains have T/E ratios in the range from 10^{-3} to 10^{-2}, whereas the corresponding eight N type

TABLE 3 *Relation between Fimbriation and Competence of Genetic Transformation in* Moraxella kingii I

Strain	Colony type II – cell line III	Degree of fimbriation IV	Competence (T/E ratio) V
4177/66	N–a	(—)*	$<3 \times 10^{-8}$
= ATCC 23330 NCTC 10529	SC a	+ +	1.1×10
CDC A170?	N a	—	$<3 \times 10^{-8}$
	N–b	—	$0(<1 \times 10^{-7})$
	N–c	—	1.7×10^{-7}
	N d	—	5.0×10^{-7}
	N–e	—	5.8×10^{-7}
	N f	—	2.4×10^{-6}
	SC–a	+ +	7.9×10^{-3}
	SC–b	+ +	1.2×10^{-2}
	SC–c	+ +	1.5×10^{-2}
	SC–d	+ +	1.5×10^{-3}
CDC 5530	N–a	—	$0(<7 \times 10^{-9})$
	(N) b	(— +)*	1.0×10^{-7}
8994/70	N–a	—	$0(<1 \times 10^{-7})$
	SC a	+	2.2×10^{-5}
9076/70	N a	—	7.5×10^{-7}
	SC a	+ +	1.9×10^{-3}

I Quantitative streptomycin resistance transformation as specified in Table 1, footnote I The transforming DNA was from various N cell lines of M kingii strains In most cases with low transformability care was taken to use autologous donor DNA i e from a mutant of the recipient cell line in addition to DNA from other strains

II SC strongly corroding typically with a wide corroding spreading zone, 8994/70 SC–a had no macroscopically visible spreading zone N non spreading generally non-corroding 4177/66 N–a exhibited a weak central corrosion beneath very large, isolated colonies after 2 days of incubation in 5530 (N) b there was a slow development of weak corrosion apparent after 1 day of incubation (9)

III Cell line designations (a–f) correspond to those used for these variants elsewhere (9, 11)

IV See Table 1, footnote IV (— +)* a moderate number of fimbriae observed in some preparations, no fimbriae detected in young cultures (about 7 hr), as those used as recipients in transformation

V See Table 1, footnote V

cell lines have an extremely low competence (or incompetence) with a maximum T/L ratio of 2.4×10^{-6} In the remaining strain (8994/70) among those differentiated into distinct SC and N types the former type has a low level competence ($T/E = 2.2\times10^{-5}$), whereas the latter is non transformable in the sensitive long term DNA exposure In the cell lines 5530 (N)-b and 4177/66 N-a very slow development of corrosion and delayed ocurrence of fimbriae (not detected in preparations from 7 hr old cultures), were observed together with extremely low competence The non fimbriated 5530 N-a however is incompetent

Of importance for evaluation of the observed T/E ratios might be differential ability of fimbriated and non fimbriated variants to form even suspensions during the transformation procedure Consequently the growth from young blood agar cultures (6–9 hr) was studied after suspension in drops of the transformation medium (with one exception mentioned below the results were identical in physiological saline) N SC and NSC cell lines of the *M nonliquefaciens* strains listed in Table 1 except 4663/62 N a and SC–a formed even suspensions Microscopically the various fimbriation forms were uniformly distributed as single cells or diplobacilli In both exceptional cases mentioned numerous small aggregates (about 10 cells each) were identically formed Also in *M bovis* (Table 2) there was no sharp distinction between fimbriation types in suspension Of the N cell lines examined in this respect 10900 N a N–b N–c 3 N–c 4 N a and 5 N–b N–c were evenly distributed whereas 3 N–a N b 4 N–b and 5 N a suspensions were partly granular like those of most SC cell lines examined Exceptionally however also SC type populations showed even distribution (*e g* 10900 SC–a 3 SC–a (not in physiological saline) and 5 SC–a) All *M kingii* SC cell lines (Table 3) formed loose aggregates in an otherwise turbid suspension This property was shared by A1702 N–b whereas the other N forms tested formed uniform turbidity, or almost so No overall correlation was therefore found in the three species between the characteristics of recipient suspensions and the T/E ratios recorded Addition of homologous DNA did not change the stabilits

Selection of Fimbriated Clones by Transformation

It was reported previously (6) that the relative proportion of SC colony forms among streptomycin resistant transformants of *M nonliquefaciens* (elicited in N type or mixed N+SC type recipients by N type transforming DNA) was high in comparison to the occurrence of SC variants among the recipient colonies This was confirmed In an experiment with 7784 N b transformed by short term exposure to 3067/66 N a DNA no signs of corrosion or spreading could be detected among a considerable number of recipient colonies whereas appr 10 per cent of the streptomycin resistant transformant colonies were strongly corroding This property proved genetically stable in the absence of DNA Among the remaining transformants, some developed small central corroding pits not seen in equally old recipient colonies One spreading corroding transformant clone was tested by electron microscopy and found to be strongly fimbriated (*Fig 21 of ref 8* whereas a non-corroding transformant alone did not reveal fimbriae In experiments with long term DNA exposure up to 100 per cent of the streptomycin resistant transformants of *M nonliquefaciens* 7784 N b and *M bovis* 10900 N a were occasionally of the SC type but in these cases with very strong selection there was also evidence of some preformed SC variants in the control recipient populations when their incubation was prolonged Most often no selection of distinct SC type was experienced in the many transformation experiments with *M bovis* N type recipient although the transformants sometimes appeared slightly more agar depressing than the recipient colonies However when N variants occasionally had become enriched in SC type populations used for transformation only SC transformants were detected at nor-

TABLE 4 *Search for Spontaneous, Incompetent Variants with Preserved Fimbriated Phenotype in High Competent SC (Spreading Corroding) Populations of Moraxellae* I

Species strain	Cell line II	Number of typical SC colonies examined	Cases of incompetence (no transformants) III
M nonliquefaciens			
4663/62	SC–a	1710	0
NCTC 7784	SC–a	830	0
M bovis			
ATCC 10900	SC–b	800	0
3	SC–a	105	0
	SC–c	769	0
4	SC–a	116	0
5	SC–b	120	0
M kingii			
4177/66	SC–a	722	0
CDC A1702	SC–a	508	0

I Semiquantitative streptomycin resistance transformation with long term DNA exposure (i e, without termination by means of DNase) to transforming DNA of the same strain or species The procedure was principally as described by *Jyssum* (12)

II See Tables 1 3

III In all cases numerous transformants were observed usually giving rise to confluent or semi confluent growth

TABLE 5 *Search for Spontaneous Variation between Competence (Low-Level) and Incompetence within Weakly Fimbriated or Non Fimbriated Populations of Moraxellae without Spreading and Corrosion (N type)* I

Species strain	Cell line II	Number of typical N colonies examined	Cases of incompetence (no transformants) III
M nonliquefaciens			
4663/62	N a	1230	1 IV
NCTC 7784	N b	1170	0
M bovis			
ATCC 10900	N–a	1164	0
M kingii			
CDC A1702	N–c	200	0
	N–d	280	0
	N–e	280	0
M nonliquefaciens			
4663/62	N b	70	70
M bovis			
3	N–a	809	809

I Long term exposure to transforming DNA from N cell lines of the same strain or species see Table 4 footnote I

II See Tables 1 3 for quantitative data on competence in the N type populations examined

III When transformants occurred their number ranged from very few to about 100 per streak depending on growth potential in relation to stage of growth at the time of streptomycin [illegible] on the T/E level in quantitative [illegible] Spontaneous streptomycin resistant [illegible] only observed as a very rare [illegible] organisms

IV This variant clone is identical [illegible] which is examined for back varia[illegible] line from the bottom of the table

mal T/E ratio in relation to the number of SC recipient cells

In *M. kingii* the selection of SC variants by transformation was often clearcut. Thus the non fimbriated A1702 N-c, N-d and N-e cell lines could develop 10–30 per cent SC type colonies among streptomycin resistant transformants when exposed without DNase termination to DNA from A1702 N-a. The observation is particularly interesting in the case of the N-e cell line, in which no SC variants were detected otherwise (9).

Generally, the selection of fimbriated clones among transformants of the various N cell lines appeared to be irregular, and the results with the same recipient could vary from experiment to experiment.

Search for Spontaneous Competence Loss or Gain Independent of Colony and Fimbriation Type Variation

Comparison of previously reported data (8, 9) with those of Tables 1–3 show that actually observed instances of colony and fimbriation type variation have always been accompanied by a considerable change of competence level, as expected from the overall association indicated between these characters. However, the number of observations in these quantitative studies is limited. In addition, the SC colony type sometimes has a rather low absolute level of competence, indicating that competence reduction occasionally takes place despite maintained fimbriation. Such reduction might possibly also occur in large steps. A search for spontaneous, large step variation in degree of competence independent of the apparently low frequent colony and fimbriation type variations was consequently performed.

In Table 4 are shown the results of attempts to detect complete loss of high level competence from single colonies with maintained SC morphology. Altogether 5680 typical SC colonies from the three species were examined by "streaking" on plates with homologous DNA, and in no instance had competence been lost, i.e., dissociated from fimbriation.

As repeatedly shown in this study, the N type colony morphology does neither distinguish between weakly fimbriated and nonfimbriated states nor between low level competence and incompetence. In a search for loss or gain of competence in various N cell lines of the three species (SC variant colonies occurring on the plates were not tested) comprising 5200 colonies (Table 5), the competence or its absence in the parent cell lines had been maintained in all but one colony

TABLE 6. *Search for Spontaneous Variation between High Level and Low Level Competence in Populations of* Moraxella nonliquefaciens *with Preserved Colony Form (SC or N Type Respectively) and Fimbriation Type*[I]

Strain	Cell line[II]	Number of typical colonies examined	Cases with high level competence[III]	Cases with low level competence or incompetence[IV]
4663/62	SC a	1126	1126*	
	N a	1136		1136
NCTC 7784	SC a	610	610	
	N b	610		610

I Long term exposure to transforming DNA from *M. bovis* 10900 N a or from *M. bovis* 3 [illegible] a. Except for such use of heterologous but related DNA (see ref. 4 for transformation compatibility between *M. nonliquefaciens* and *M. bovis*) the methodology was principally as described for competence screening with homologous DNA (12).

II See Table 1 for quantitative data on transformability of the cell lines in homologous reactions.

III Numerous transformants (from 80 to innumerable per "streak").

* None of 10 N variant colonies occurring in the SC populations at the same time revealed high level competence.

IV Few (one to nine) or no transformants.

(out of 1230 examined of the cell line in question) The variation was from low level competence to incompetence, and it was accompanied by an apparently further reduced ability to form occasional fimbriae (see footnote IV of Table 5, and Table 1)

Finally, attempts were made to detect variation between high level ($T/E \sim 10^{-2}$) and low level ($T/E \sim 10^{-5}$–10^{-6}) competence in either direction in SC and N cell lines of two strains of *M nonliquefaciens* (Table 6) Again the search was for spontaneous variation of competence dissociated from the colony and fimbriation type typical of the competence level in question In 3480 colonies examined no such independent large step competence variation was detected On the other hand competence variation associated with colony and fimbriation type was detected without difficulty in the same series of experiments (see footnote III of Table 6)

DISCUSSION

The present report confirms and extends our previous brief reports (6, 7) on association in moraxellae between the presence of fimbriae and enhanced competence of the cells as shown in streptomycin resistance transformation under presumed optimal conditions The association is documented for a number of strains and variants in three different species, one of which is genetically distant from the other two more closely related ones In addition to the correlation with competence fimbriation is associated with the spreading corroding mode of colony growth, formation of a surface pellicle in fluid medium and the ability of individual cells to move by "twitching motility" on agar surface (5 8 9 11)

The increase in competence accompanying fimbriation regularly amounts to 400–1000 fold or more when comparing cell lines apparently differing only in fimbriation and colony type This increase must be considered to be far in excess of experimental variation such as could conceivably be explained by different degree of clustering of cells slight differences in growth phase etc

The uptake, integration and expression of foreign genetic material in bacteria constitute a complex multistep process (10, 17, 19) Evidently, each step may be influenced positively and negatively by a number of cellular factors Our working hypothesis is at present, that the fimbriae observed may be concerned with the early uptake mechanisms of DNA, because of their obvious extracellular appearance giving the fimbriated cells quite distinct surface properties as compared with their non fimbriated counterparts It could also be, however that the fimbriae are functionally inert in this respect, only reflecting the presence of (a) competence factor(s) The often but somewhat irregularly, observed emergence of spreading corroding (SC) colonies in streptomycin resistance transformed offspring of non corroding (N) colonies confirms the advantage of fimbriated cells for transformation This selection can be explained by the presence of a low number of highly competent SC type cells in the mixture of recipient cells

On the other hand it must be kept in mind that a low level of competence can be found in non fimbriated N recipients without such selection (one cannot exclude the possibility that these have short fimbriae transiently, however) and also that the presence of fimbriae does not necessarily lead to a high level of competence

Apart from our own previous reports, we are not aware of any other publication where fimbriation is linked with competence in genetic transformation The well known association of sex fimbriae with sex factors (F colicinogenic and resistance transfer factors) and the transfer of genetic material by bacterial conjugation is a parallel of certain interest (1 10) The common fimbriae e g type 1 fimbriae of enterobacteria have also been shown to greatly enhance the speed of spread of the *col I* factor (14 15) However in none of these cases the fimbriae have been interpreted as organelles of importance for recipient ability *per se* only as possible mediators of genetic transfer *from* the fimbriated cell or of cell contact during

conjugation. The presumed injection of DNA through fimbriae by some bacteriophages (1, 10) is the only other example of possible centripetal transfer of genetic material associated with fimbriae. Anyway, we think all these reports taken together strongly point toward roles of fimbriae in intercellular transport, perhaps particularly of nucleic acids.

When considered together with data on variation of colony and fimbriation types (5, 8, 9), this report shows that, generally, the spontaneous occurrence of large step variation of competence in moraxellae is infrequent and the emergence of incompetent variants extremely so. When young colonies were spread for analysis in the previous studies, the emergence of N variants within SC colonies was observed only occasionally among thousands of the progeny. When typical SC colonies were directly streaked on plates with transforming DNA for detection of spontaneous, complete loss of competence, the results were always negative. In the study of N cell lines only one case of spontaneously completely lost low-level competence was detected (less than 0.1 per cent) and no case of variation to high competence when not associated with the (apparently also infrequent) N to SC type variation.

Our results on the stability of competence appear in some respects to be different from what reported by *Jyssum* & *Lie* (13) for certain strains of *Neisseria meningitidis*. The latter authors described spontaneous clonal emergence of variants with complete permanent loss of competence with a frequency of 0.4 to above 99 per cent. In a later study *Jyssum* (12) described a variant type with low transformability arising from clones with high-level competence. This intermediate type usually reverted to high level competence with very high frequency and lost competence somewhat less frequently.

The fimbriae associated variation of competence in our studies has been found to be bidirectional between high and low levels. It is possible, however, that some occasional N variants will prove to be irreparably non-fimbriated and at the same time permanently incompetent. For the time being the genetic mechanism of competence variation in these bacteria is unknown, as it is for variation of fimbriae associated colony type (8).

The very recent report by *Swanson et al.* (18) on fimbriation of certain colony forms of *N. gonorrhoeae*, which probably are identical to those distinguished by high transformability in the independent studies of *Sparling* (16), strengthens the prediction (7) that an association between fimbriation and competence will be found in this species, when directly tested for. There are therefore strong indications that the association discovered is of general importance.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

I The Usefulness of Trimethylsilyl- and Trifluoroacetyl Derivatives for Strain and Species Characterization

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Gas liquid chromatography (GLC) of trifluoroacetylated (TFA) whole cell methanolysates as a procedure for obtaining species specific GLC elution profiles has been introduced This method and a modification of the previously described trimethylsilyl (TMS) derivatization used for identical purpose were tested on four strains belonging to the bacterial species *Neisseria catarrhalis*, *N ovis* and *N meningitidis* The TFA and TMS elution profiles are compared and their usefulness in characterization of strains and species in relation to the best reproducibility obtainable is discussed The TFA method gives better resolved and more reproducible elution profiles The method appears to have a high potential as an aid in bacterial classification and identification

Direct use of gas chromatograms as finger prints for classification and identification of bacteria is a recent and promising application of gas liquid chromatography (GLC)* *Abel et al* (1) extracted and transesterified lipids from selected species of various bacterial families and they obtained characteristic GLC elution patterns of the fatty acid methyl esters *Yamakawa & Ueta* (20 22) introduced fragmentation of dried cells by HCl catalyzed methanolysis Two main groups of substances were released by this treatment methyl esters of fatty acids and methyl glycosides of various monosaccharides Extraction of the unpolar fatty acid methyl esters by light petroleum and trimethylsilyl derivatization of the remaining methylglycosides prior to GLC gave them two sets of elution profiles from selected species of neisseriae and the family Micrococcaceae *Farshy & Moss* using a similar but simplified technique obtained species specific elution profiles within the genus *Clostridium* (11, 12) Direct GLC of pyrolyzed bacteria is another much used technique *Reiner et al* obtained reproducible elution profiles distinguishing between mycobacterial types (18) GLC analysis of volatile metabolites (headspace gas) and extracted derivatized metabolites from spent medium also has been reported to give information of

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* The following abbreviations are used
- BSA N O bis trimethylsilyl acetamide
- GLC Gas liquid chromatography
- TFA Trifluoroacetyl
- TMS Trimethylsilyl

value in classification and identification (14)

The published reports show that GLC methods are promising tools in taxonomic work, although they have not been fully evaluated and exploited in this respect. We therefore decided to perform an investigation, by means of GLC, on species and groups of moraxellae and neisseriae. These bacteria were chosen because 1) the relations between them on the nucleic acid level are well known from previous studies, thus giving a rational basis for evaluation of the GLC methods applied, and also because 2) some still unsolved classification problems exist in these groups that might be elucidated by GLC in combination with the data on nucleic acid homologies and heterologies (see *Froholm et al* (13) and *Bovre et al* (7) for references and discussion)

Methanolysis fragmentation followed by volatilization and stabilization by a suitable derivatization reagent prior to GLC, were chosen for the present study. Highly specific and relatively simple elution profiles of various bacterial strains are obtained using these methods (11, 12) and conventional GLC can be used. However, the usefulness of a "finger print" of this type is rather restricted if time to time variations are large. Before starting extensive taxonomic studies based on GLC elution profiles we, therefore, felt the necessity to closely examine the reproducibility of the chosen methods under standard conditions. Development of the technique described is based on the methods reported by *Yamakawa & Ueta* (22) and by *Farshy & Moss* (12). Other derivatization reagents and technical modifications giving simpler and less sample demanding procedures have been introduced. The reproducibility of two types of elution profiles will be discussed. Presentation of the taxonomic data will be given in following reports (7, 13)

MATERIALS AND METHODS

Bacterial Strains

*Neisseria** *catarrhalis* Ne 11, *N.** *ovis* 199/55 and 37/59 and *N. meningitidis* B8152/66 were selected for this part of the studies. The taxonomic interrelations of these species (and strains) have previously been studied by one or more of the methods genetic transformation (3, 4, 9), DNA base ratio determination (6), and pulse RNA-DNA hybridization (5). Their cultural and biochemical behaviour has been examined (3, 4)

Media and Growth Conditions

Standard medium: blood agar (routine medium for primary culture of clinical specimens at Riks hospitalet, Oslo) consisting of Tryptose Blood Agar Base (Oxoid), dextrose 0.1 per cent, human citrated blood 5 per cent, pH 7.6. These media were also tested: Tryptose Blood Agar Base (Oxoid), pH 7.2 and Mueller Hinton Medium (Difco), pH 7.4. The organisms were grown at 33° C for 20 hours in a humid atmosphere. *N. meningitidis* was also examined after incubation at 37° C with transient initial exposure to a small amount of CO_2 (shown in Fig 2). The two conditions of incubation did not influence the elution pattern, as was also shown for *N. elongata* (7)

Harvesting was routinely done by washing the bacterial colonies off the plates with a right angled glass rod with the aid of a 0.5 M phosphate buffer, pH 7.2. This technique is simple, rapid and gives remarkably low media component background (Fig 1). Usually one plate gives sufficient material for at least 12 chromatograms. The cells were centrifuged at 3000 × g for 20 minutes, washed twice with distilled water, centrifuged and freeze dried. The dried material was stored under nitrogen in carefully closed vials at −20° C

Methanolysis

Freeze dried bacterial cells (2-10 mg) and 1N HCl in methanol (1 ml, both components carefully dried) were heated under nitrogen in 10 ml glass tubes with Teflon lined screw caps at 90° C for [illegible] hours. The cooled heterogeneous mixture was centrifuged at 1000 × g for 7 minutes. After one washing of the residual material with 1 ml of dry methanol the supernatants were pooled, followed by concentration to dryness on a rotary evaporator at 25° C. To remove the last traces of acid and water, the samples were evacuated for 2 hours at 25° C with an oil pump. The residue was dissolved in 0.1 ml anhydrous pyridine (distilled and stored over molecular sieve 4A, Merck), flushed with nitrogen, sealed and stored at −20° C

* The *catarrhalis* and *ovis* species are temporarily assigned to genus *Neisseria*, although both *Moraxella* and *Branhamella* have been proposed as genus designations for these organisms (8, 15)

Trimethylsilyl (TMS) Derivatization

To 20 µl of the pyridine solution described above, 30 µl of a mixture containing 1 per cent trimethylsilyl-chlorosilan (Merck) in N,O-bis-trimethylsilyl-acetamide (BSA) (Merck) was added. After a minimum of 12 hours in a tightly stoppered vial at room temperature the reaction was completed and samples were injected directly onto the gas chromatograph.

Trifluoroacetyl (TFA) Derivatization

Twenty µl of the methanolysate-pyridine solution described above was concentrated to dryness on a rotary evaporator at 25° C. 50 µl of trifluoroacetic anhydride (Koch-Light)/acetonitrile (Koch-Light) (1:1 v/v) was added to the residue. After careful heating to boiling with a hairdryer, the vial was stoppered and left at room temperature for 1–2 hours before injection on the gas chromatograph.

Gas-Liquid Chromatography (GLC)

We used a Hewlett-Packard model 5750 equipped with dual flame ionization detector and 2 m long glass columns of 2 mm inner diameter. The detector temperature was kept at 250° C and the injector block at 230° C. Nitrogen with a flow rate of 36 ml/minute was used as carrier gas. TMS derivatized methanolysates were analyzed according to *Farshy* & *Moss* (12) on a column packing consisting of 3 per cent OV-1 on Gas-Chrom Q, 100/120 mesh (Applied Science Lab. Inc.). The column temperature was kept for 5 minutes at 130° C, then programmed to 240° C at 4° C per minute. For the TFA derivatized methanolysates, a 10 per cent UCC-W982 liquid phase on Chromosorb W, 100/120 mesh (AW-DMCS-HP) (Hewlett-Packard) was used as stationary phase and support. The selected temperatures were: first 4 minutes at 100° C, then programmed 6° C/minute to 210° C.

Estimation of Reproducibility

The reproducibility of the described methods was evaluated on the basis of peak height variations encountered when one particular bacterial specimen was repeatedly "fingerprinted" by the technique (see Results and Discussion).

Two sets of experiments were performed:

1. Registration of peak height variation caused by small uncontrollable dissimilarities in reaction conditions, gas chromatography and recording. The experiment included a comparison of the TFA and TMS procedures (Table 1).
2. Registration of peak height variations experienced when the complete TFA procedure was taken into account, including influences of time to time variations of standard medium composition, growth conditions, harvesting and storing (Table 2).

In both experiments three samples of arbitrarily chosen bacterial strains (*N. ovis* 37/59 and *N. catarrhalis* Ne 11, respectively) were methanolyzed and derivatized according to the described methods, and three parallels of each sample were chromatographed. The bacterial samples used in experiment 1 were from the same incubation and the three used in experiment 2 were taken from three different batches of the same bacteria (grown on standard medium and harvested by standard technique but at various times). Seven distinct peaks in the elution profiles were selected for the estimation of variation (Figs. 3 and 4). Influence of casual variations in concentration, injection volume and detector sensitivity was reduced to a minimum by transforming the measured values to per cent peak height.

Standard deviation σ and relative standard deviation σ_{rel} (per cent of the arithmetic mean $\bar{x}$) were calculated according to established formulae (10) and listed in Tables 1 and 2. Reliability interval, usually defined as $\bar{x} \pm 2\sigma$, is a very practical measure of reproducibility as it covers 95 per cent of all observations (valid only when the number of observations are large and when the values fit a Gaussian distribution) (10). The reliability intervals presented as the seven vertical lines in Figs. 3 and 4 are calculated from the percentage values $\bar{x} + 2\sigma$ and $\bar{x} - 2\sigma$ of the corresponding seven peak heights in the three chosen chromatograms. They are subject to many approximations but in our opinion still of value for estimation and illustration of reproducibility.

RESULTS AND DISCUSSION

When using a rich and solid medium and a growth period of only 20 hours the cell population is considered to be relatively homogeneous with a low percentage of dead cells. Variation in chemical composition of cell constituents likely to be depolymerized by methanolic hydrochloride (as membrane/cell wall glycolipids) is also hopefully kept at a minimum by careful standardization of medium composition and growth conditions (within the practical limits of laboratory routine). Comparison of elution profiles of *N. catarrhalis* Ne 11 grown on the three different media (see Materials and Methods, one single experiment) indicated that the influence of medium composition on appearance of the profiles is *significant* but not at

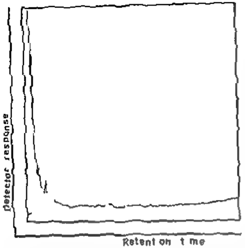


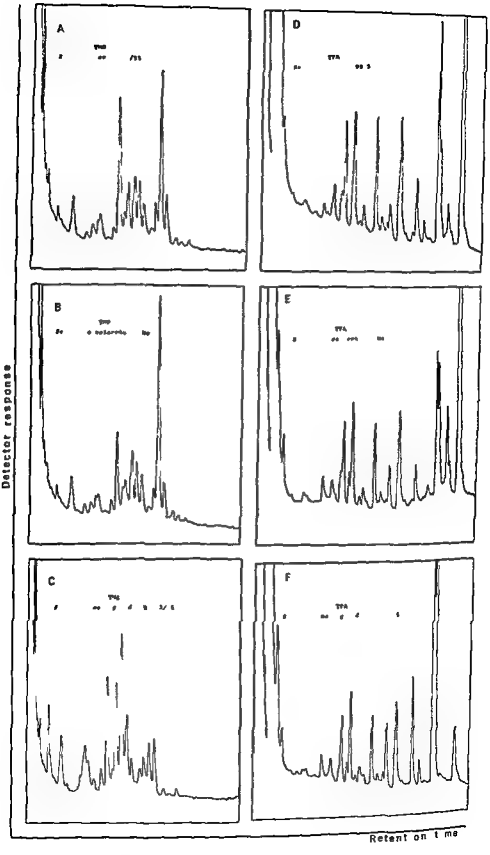
Fig 1 Medium component background Forty uninoculated standard medium plates were incubated at standard conditions and harvested by the standard procedure (see experimental part) The residual material obtained was methanolyzed derivatized and chromatographed as a standard 3 mg sample See text and Fig 4 for the chromatographic conditions

all dramatic if the growth rate is normal Harvesting the cells by washing them off the medium surface with a buffer is a rapid and simple technique No significant contamination with medium components is detected (Fig 1)

Treatment of bacterial cells with hydrogen chloride in methanol under controlled anhydrous conditions gives a very mild fragmentation About 50 per cent of the material dissolves leaving an almost colourless supernatant after centrifugation Proteins and nucleic acids will not hydrolyze by the reaction and remain insolubilized The linkages in lipo polysaccharides triglycerides and other membrane/cell wall structures are far more acid labile and possibly more exposed to the reactants Various fatty acid methyl esters and various monosaccharide methyl glycosides are therefore expected as the main constituents in the volatilizable part of the methanolysate fraction (22) Small variations in reaction conditions like acid strength reaction time and temperature do not influence the elution pattern significantly (11 unpublished results)

The TMS method used is a modification of the one described by *Farshy & Moss* (12) BSA is reported to be a more powerful TMS donor (16) and to give higher yields of completely derivatized amino and hydroxyl groups compared to the hexamethyldisilazane/trimethylchlorosilane reagent and was therefore chosen Trifluoroacetic anhydride has for some time been used as an alternative volatilization reagent to the TMS donors in GLC analysis of compounds with hydroxyl and/or amino groups (2, 19 21) Trifluoroacetic anhydride in acetonitrile gives in a few minutes a completely derivatized monosaccharide (21) and the formed ester and/or aminoacyl compounds have a remarkably high volatility and are relatively stable The composition of a TFA derivatized *N catarrhalis* methanolysate did not change significantly after six days storage in a tightly stoppered vial at room temperature (unpublished results) Various silicone liquid phases and other chromatographic parameters were tested out for TFA derivatized methanolysates A stationary phase consisting of a 10 per cent methyl vinyl silicone polymer (UCC W982) on a silanized acid washed Chromosorb W gives well resolved elution profiles in about 40 minutes (Figs 2–4)

Figs 2 and 3 show pairs of representative TMS and TFA elution profiles of the four bacterial strains Both types of profiles consist of a similar number of peaks but the peaks in the TFA profiles are sharper and better resolved and therefore more detailed and easier to interpret Seven of the most distinct peaks in the two types of chromatograms were chosen for reproducibility estimation and comparison The numbering of the peaks is based on the sequence of elution in the two chromatographic systems There is no known chemical relationship between TMS and TFA peaks with identical number (Fig 3) The reproducibility obtained by the two methods was estimated as outlined in the experimental part and the result is presented in Table 1 Some substances in the derivatized methanolysates are obviously more sensitive to small experimental variations than others


A
B
C
D
E
F
Detector response
Retent on t me

Thus, the heights of peak 1 and 7 in the TFA elution profiles and of peaks 2 and 7 in the TMS profiles show the highest degree of variation (see discussion of reproducibility) Generally, however, the peak heights are remarkably constant (Table 1 and Fig 3), five peaks in the TFA profile having a σ_{rel} in relative peak height below 3 0 The relative standard deviation values of comparable peaks in the TMS profiles are higher and as a result we at present consider the TFA profiles to be more detailed "finger prints" and easier to reproduce Use of a more volatile TMS donor system like bistrimethyl silyl trifluoro acetamide in acetonitrile and a higher percentage of a silicon polymer in the stationary phase system may, however, result in considerably better TMS profiles (unpublished results) Preparation of both a TFA and a TMS elution profile from each methanolyzed bacterial sample will of course give more information and thereby more reliable results

The bacterial 'finger print', or elution profile, obtained by the GLC technique is a continuous record of detected substances in the derivatized methanolysate eluted from the column It is characterized by 1) the number of peaks recorded (number of substances) 2) their relative quantity (relative peak heights) and 3) their time of elution (retention time) Lack of reproducibility will be observed as time to time variation of these three parameters when a specific bacterium is repeatedly '*fingerprinted* A careful examination of many "finger prints' from closely related bacterial strains revealed that apparently identical peaks can be found in the chromatograms with only minor variations in relative retention time (7, 13) The relative peak heights, on the other hand, do show differences In extreme cases, peaks that are prominent in some strains may be lacking altogether in others As a reasonable approximation we therefore decided to estimate reproducibility from peak height variations only By using modern gas chromatographic equipment, standard injection technique and careful calibrations of gas flows and temperatures, the time to time variations caused by instrumental parameters are well known (17) and they are considered to give a small contribution to peak height variation* The reliability of the chromatographic system in the present study is well documented in Table 1 the variation in relative peak heights within one sample is remarkably small (with a few exceptions close to the practical limits of precise peak height measurement)

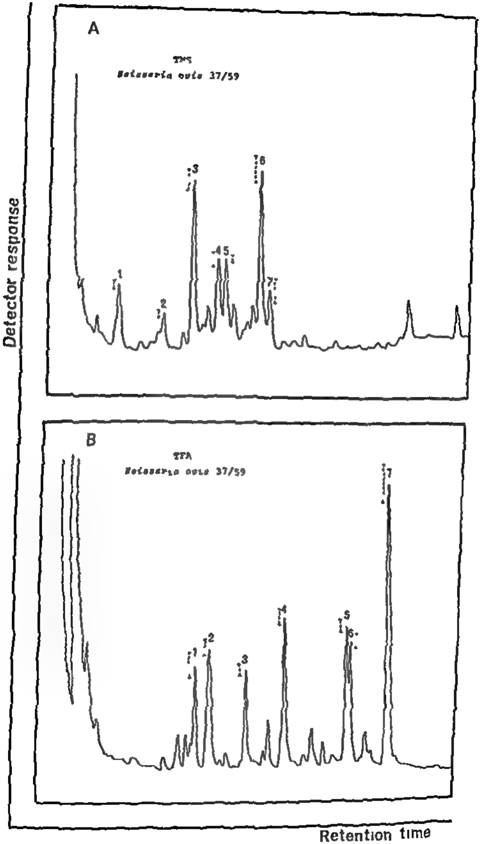
The three samples of *N ovis* 37/59 used to obtain the results given in Table 1 are from the same incubation They have been handled in the same way, by the same reagents at the same time and are chromatographed in sequence The tabulated peak height differences, illustrated in Fig 3 are therefore considered to be mainly due to uncontroll

Fig 2 Typical TMS and TFA profiles Methanolysates from three *species of neisseriae* obtained as outlined in the text were derivatized and chromatographed according to the *described* TMS and TFA procedures. See text and Fig 4 for the chromatographic conditions

* Two important factors should always be kept in mind in relation to the chromatographic setup

1) The retention time is relatively sensitive to variation in temperatures carrier gas flow rate and liquid phase concentration (age of column) Time to time variation of these parameters is, within certain limits uncontrollable and small variations in retention times must be expected Differentiation of bacterial specimens based on peaks with small differences in retention times must therefore, be avoided unless careful controls are used such as internal standards and peak identification techniques like mass spectrometry

2) Bacterial methanolysates will always contain some nonvolatilizable material Repeated injections of such samples result in accumulation of a tar like residue in the first five centimeters of the column This material after growth beyond a certain limit will influence carrier gas flow rate, the separation properties of parts of the liquid phase surface and the 'on column degradation' of the sample A satisfactory reproducibility, therefore, requires removal of this material after a certain number of injections (normally about 50–100)



A
TMS
37/59
1
2
3
4
5
6
7
B
TFA
37/59
1
2
3
4
5
6
7
Detector response
Retention time

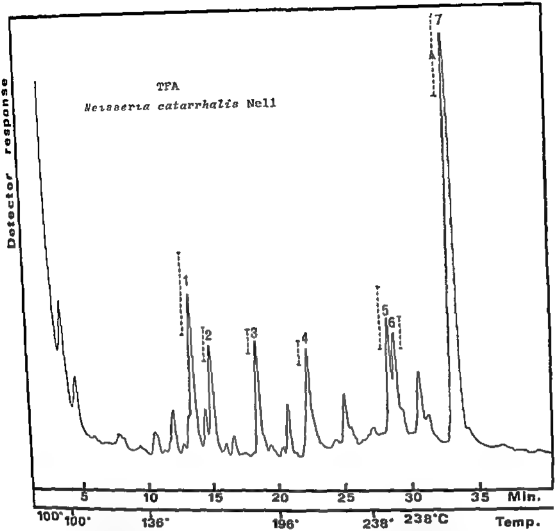


Fig 4 Peak height variation of individual TFA "finger prints" caused by random errors in the entire procedure (including growth, harvesting and storing for a short time, illustated as reliability intervals (see text for definition and discussion) Three samples of *N catarrhalis* Ne 11 (three different incubations harvested by a few weeks intervals) were methanolyzed, TFA derivatized and chromatographed in sequence, three parallels of each sample The broken lines illustrate the reliability intervals of the seven selected peaks (see text and Table 2) The gas chromatographic separation was performed on a 2m by 2mm glass column filled with Chromosorb W (AW DMCS HP, 100–120 mesh) coated with 10 per cent UCC-W982 Nitrogen flow rate 36 ml/min Gas chromatograph Hewlett-Packard 5750 equipped with flame ionization detector Electrometer settings range 10^2, attenuation 4 or 8 Temperature was first kept 4 min at 100° C, then programmed 6° C/min to 240° C and held isothermally at 240° C

Fig 3 Peak height variation of individual TMS and TFA "finger prints" caused by random errors in methanolysis, derivatization, chromatography and registration (not growth, harvesting and storing), illustrated by reliability intervals (see text for definition and discussion) Three samples of *N ovis* 37/59, one incubation, were treated by the same reagents at the same time and chromatographed in sequence, three parallels of each sample The broken lines illustrate the reliability intervals of seven distinct, arbitrarily chosen peaks (see Table 1) See text and legend to Fig 4 for the chromatographic conditions

TABLE 1* *Comparison of TFA and TMS Elution Profiles by Relative Peak Height Variation Measurements*

	Relative Peak Height§						
Peak no	1	2	3	4	5	6	7
Retention time† (sec)	826 (819–835)	907 (901–915)	1111 (1106 1119)	1333 (1326–1342)	1691 (1681–1703)	1714 (1703–1726)	1942 (1926–1961)
TFA sample no							
1	16 2 15 8 15 9	16 2 16 9 17 3	12 8 13 0 12 4	20 6 20 4 20 3	18 2 17 9 18 0	15 9 16 1 16 2	/ 37 3
2	15 2 14 9 16 1	16 8 17 1 16 9	12 7 12 7 13 2	20 2 20 1 20 1	18 2 18 0 17 1	17 0 17 1 16 2	35 7
3	14 0 14 5 14 5	16 3 16 1 16 0	13 4 13 5 13 1	20 9 20 7 20 8	18 7 18 5 18 5	16 7 16 7 17 2	39 2 38 6 37 9
x*	15 2	16 6	13 0	20 5	18 2	16 6	37 2
σ‖	0 81	0 48	0 36	0 31	0 39	0 48	1 82
σrel"	5 3	2 9	2 8	1 5	2 1	2 9	4 9

Peak no	1	2	3	4	5	6	7
Retention time (sec)	316 (311–323)	566 (559–576)	731 (725–741)	872 (860–877)	913 (906 923)	1106 (1096–1118)	1159 (1151–1172)
TMS sample no							
1	10 0 9 9 9 7	5 5 5 4 5 3	25 6 26 9 26 7	12 9 12 4 12 3	12 3 12 1 12 4	27 5 25 8 25 3	6 3 7 5 8 3
2	10 0 10 0 9 8	4 9 5 1 5 0	28 0 27 6 27 8	12 4 12 2 12 0	12 4 12 5 12 0	26 3 26 6 26 3	5 9 6 0 7 0
3	10 7 10 3 10 2	5 6 5 6 5 5	27 6 28 0 28 1	13 4 13 3 13 5	12 4 12 9 12 7	24 9 24 1 24 5	5 1 5 7 5 6
x	10 1	5 3	27 3	12 7	12 4	25 7	6 4
σ	0 30	0 26	0 83	0 57	0 28	1 10	0 98
σrel	3 0	4 9	3 0	4 5	2 3	4 3	15 3

* Three samples of lyophilized cells (*N avis* 37/59 from one incubation) were methanolyzed and derivatized Three parallels of each sample were chromatographed in sequence (see text for details) The height of seven distinct peaks (see Fig 3) were measured, transferred to per cent values and tabulated

§ Per cent of the total height of the seven selected peaks (for the TFA samples of the first six peak heights)

† Mean value the numbers in parantheses show range

* Mean value

TABLE 2* *Time to Time Variations of Individual TFA Finger Prints*

Peak no	Relative Peak Heights§						
	1	2	3	4	5	6	7"
Sample*							
1	22 5 21 4 23 0	14 8 15 0 15 0	16 0 15 4 15 6	15 6 14 7 15 3	16 7 16 6 16 1	14 5 14 6 14 9	58 9 53 0
2	29 0 28 0 26 6	13 5 12 7 12 9	18 1 16 9 16 2	13 6 14 5 14 8	13 2 14 9 15 8	12 6 13 1 13 7	45 1
3	28 0 27 4 27 8	12 4 12 3 12 0	16 3 16 7 16 4	16 1 15 7 17 3	11 9 12 2 11 3	15 3 15 8 15 2	46 3 48 8 46 5
x†	26 0	13 4	16 4	15 3	14 3	14 4	49 8
σ†	2 85	1 22	0 80	1 06	2 16	1 07	4 82
σrel†	11 0	9 1	4 9	6 9	15 1	7 4	9 7

* Three samples of lyophilized cells (*N catarrhalis* Ne 11 from three different incubations) were methanolyzed and TFA derivatized and chromatographed in sequence (see text and Table 1)

§ Relative to the sum of the first six of the seven selected peak heights (see Fig 4)

† See Table 1 and text for explanation

" Open space peak out of scale

able experimental (random) errors, and the reliability intervals (see experimental part for definition) as an adequate illustration of the best reproducibility by this 'fingerprinting' procedure of lyophilized bacterial cells.

Information of the specific influence of small time to time variations in factors like standard medium composition, growth conditions, harvesting procedure, freeze drying and storing on the 'finger print' appearance is rather difficult to obtain because the impossibility of varying one of the parameters at a time. However, an indication of the overall influence of these parameters on peak height may be obtained. For this purpose, three samples of *N. catarrhalis* Ne 11 grown at three different times with a few weeks intervals were harvested, freeze dried and stored by standard methods, then methanolyzed and derivatized at the same time by the same reagents and chromatographed in sequence. The resulting peak height variations are tabulated in Table 2 and illustrated in Fig. 4. These results in relation to Table 1 indicate that time to time variations of the procedure prior to methanolyzation contribute significantly to peak height variations.

The potential taxonomic usefulness of whole cell methanolysate "finger prints" is well illustrated. There is a marked similarity between the corresponding elution profiles from two independent isolates of *N. ovis* (Figs. 2A, 2D, 3A and 3B). The clear differences between the chromatograms of *N. meningitidis* (Fig. 2, C and F) and *N. ovis* are also significant and both observations point to the use of the methods for identification of bacterial isolates. On the other hand, the chromatographic patterns of *N. catarrhalis* (Figs. 2B, 2E and 4) and *N. ovis* do not appear significantly different. The two latter species are however closely related species as shown by their nucleic acid compatibility, whereas *N. meningitidis* is a genetically very distant organism (4, 9). Therefore, the GLC technique used appears to distinguish groups of organisms and it seems justified to conclude that the approach has a high potential in bacterial classification. In the following papers (7, 13) the technique will be critically tested on organisms with high, as well as low, genetic interrelationship.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

II *A Taxonomic Evaluation of the Method for Species of* Moraxella

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Twentyfive strains or substrains belonging to the bacterial species *Moraxella nonliquefaciens, M bovis, M kingii, M osloensis, M phenylpyrouvica* and *M urethralis* (tentative designation) were examined by gas liquid chromatography (GLC) of whole cell methanolysates Each strain gave a reproducible chromatogram, usually with no difference between non fimbriated and fimbriated variants With a few exceptions there was no significant difference between the elution profiles of strains belonging to the same species. The study revealed similarities as well as distinct dissimilarities in interspecies comparisons When compared with *M bovis* the species *M nonliquefaciens* and *M phenylpyrouvica* showed most similarity whereas *M kingii* revealed the greatest chromatographic deviation The remaining two species were intermediate in this respect The results are discussed in relation to taxonomic data previously arrived at by other methods in particular measurements of nucleic acid homologies and heterologies Generally the two groups of results were consistent and it is concluded that GLC is a valuable tool for evaluation of relationship between species adding significantly to the reliability of species allocation to genus GLC appears also applicable for species identification of isolates of moraxellae and similar organisms However, the sometimes great similarities between closely related species and the occasionally observed (minor) intraspecies variations in elution profile substantiate the need for supplementary methods

Three species of moraxellae have recently been investigated with respect to colony morphology variation in relation to the presence of fimbriae (7, 9 14) 'twitching motility' of the cells on agar surface (15) and competence in streptomycin resistance transformation (10) When the methods of gas-liquid chromatographic (GLC*) analysis became available in our laboratories we decided to perform a GLC study of the bacterial varieties The initial results were quite encouraging from a taxonomic point of view (see also ref 19), and three additional species of rodshaped oxidase positive organisms were included to allow a limited taxonomic evaluation in the genus *Moraxella* In an accompanying paper (13) the results of a

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* The following abbreviations are used
GLC = Gas liquid chromatography
TFA = Trifluoroacetyl
TMS = Trimethylsilyl

similar study in groups of neisseriae are presented and discussed in relation to those of the present report

MATERIALS AND METHODS

Bacterial Strains and Variants

Twentyfive strains or variants were examined of the species *Moraxella nonliquefaciens*, *M bovis*, *M kingii* *M osloensis*, *M phenylpyrouvica* and *M urethralis* (tentative designation) The specimens included are listed in Table 1 Of the species *M nonliquefaciens*, *M bovis* and *M kingii*, both non corroding non fimbriated or weakly fimbriated (N) as well as spreading corroding, strongly fimbriated (SC) variants were studied from most strains In addition to conventional biochemical and cultural identification all the strains had previously been found representative of their respective species designations by genetic transformation or DNA base ratio determination most often in combination (see references in Table 1) By such approaches and by means of pulse RNA DNA hybridization, the interspecies nucleic acid compatibilities of these organisms have been established (see the section of Discussion)

Cultivation and Gas Chromatography

The methods of cultivation on standard blood agar medium (for 20 hr at 33° C in a humid atmosphere without CO_2 supplementation), harvesting the cells, and gas chromatography, were as detailed in the preceding publication (19)

RESULTS

Representative GLC elution curves of TFA derivatives of each of the species investigated are shown in Fig 1 (see also ref 13) Two strains each of *M nonliquefaciens* and

TABLE 1 *Species, Strains and Variants of Moraxellae Examined by Gas Chromatography*

Species	Strains	Variants*	References
M nonliquefaciens	4663/62 = ATCC 19975	N a	
	= NCTC 10464 (type strain)		1, 2, 7 8 9,
	NCTC 7784	N a, SC a	10, 11
	3067/66	N a, SC a	
M bovis	ATCC 10900 (type strain)	N a	
	3	N a, SC a, SC c	1, 3, 8, 9,
	4	N a, N b, SC a	16, 21
	5	N b, SC a	
M kingii	4177/66 = ATCC 23330	N a, N b SC a	
	= NCTC 10529 (type strain)		8, 14 17
	9076/70	N a SC a	
M phenylpyrouvica	A1920 = ATCC 19976		
	= NCTC 10465 (type strain)		4, 8 11
	5873		
M osloensis	2863 = ATCC 23333		
	= NCTC 10526 (type strain)		
	ATCC 17958 (*Moraxella polymorpha*		8 12
	Flamm 1957 strain 1078/55)		
M urethralis	WM6		
(tentative designation)	WM20		20

* N and SC symbolize colony morphology types, N type cells are either non fimbriated or may have occasional fimbriae whereas strong fimbriation characterizes the SC type. The labels a-c indicate separate isolates of the respective colony morphology from the individual strain (refs 9, 10, 14)

M bovis are presented All are shown in a similar manner, and they can be directly compared with the chromatograms shown in the preceding report (19) and in the following one (13) In the upper part of each chromatogram are given the retention times determined for each of seven seemingly identical peaks characterized in the preceding paper (19), and lower down the retention times for peaks found by interpolation (see the section of Discussion)

Based on Table 2, TFA derivative chromatograms of all strains will be analysed in a comparative manner First, strains and varieties within the different species will be characterized and compared before the species are compared with each other After several trials it was decided to choose strain 10900 of *M bovis* as a common denominator strain for comparisons When another strain (of the same or different species) was to be compared with strain 10900, it was first decided which ones of the seven selected peaks had about the same relative height distribution pattern in those two strains (reference peaks) Three to six reference peaks were found to be a satisfactory number, allowing for the experimental variation to be expected (19) All the peaks in the other strains examined were evaluated by pair wise comparison with *M bovis* 10900 the peak height being expressed as an approximate percentage of the seemingly identical peak in the latter strain The reference peaks served to provide a correction factor if they were of a different magnitude in the two strains compared

The upper part of Table 2 shows which ones of the selected peaks served as references when the chromatograms were analysed and tabulated (Table 2 lower part) These peaks as well as every listed peak in the reference chromatogram (strain 10900) were for convenience given a relative peak height value of 100 In this part of the table all intermediate peaks discussed are entered in the leftmost column with suitably rounded off retention time values It is impossible to evaluate all these peaks in every strain examined and the table contains only the ones that are easy to recognize We want to stress that we do not think the peaks with identical retention times necessarily contain the same substances (see also Discussion) The difficulty of reproducing retention times has been emphasized before (19) and should always be kept in mind Due to the known peak height variation with experimental conditions (19), the peak height per cent values given for some of the peaks in individual experiments are inaccurate For the present comparisons, it is assumed that up to ± 30 per cent peak height difference might be due to methodological variations (growth conditions, storage *etc*), in extreme cases Still the values as they are given seem to point to important common features as well as to striking differences which will be detailed below

Variation within Species

M nonliquefaciens Three strains and a number of different variant isolates were studied (see Table 1) The elution profile of 7784 N-a is shown in Fig 1C and that of 4663/62 N-a in Fig 1D The fimbriated (SC) and the non fimbriated (N) varieties of strain 3067/66 showed quite identical patterns, whereas 7784 SC a revealed somewhat reduced peaks with 1691 and 1714 sec retention time as compared to the N a variant of the same strain Table 2 shows the strains 4663/62, 7784 and 3067/66 to be rather similar with respect to most peaks There are however some small and reproducible differences like the 1075 peak evident in strain 4663/62 and the low 1370 peak of this strain

Fig 1 *TFA elution profiles of moraxellae* Lyophilized bacteria were methanolyzed trifluoroacetylated and gas chromatographed (19) The elution diagrams shown are from three different harvests analysed at different times but under identical chromatographic conditions In the upper part of the chromatograms are given normalized retention times for seven comparable peaks (see the sections of Results and Discussion) and lower down retention times for other peaks discussed in the text † Tentative designation (20)

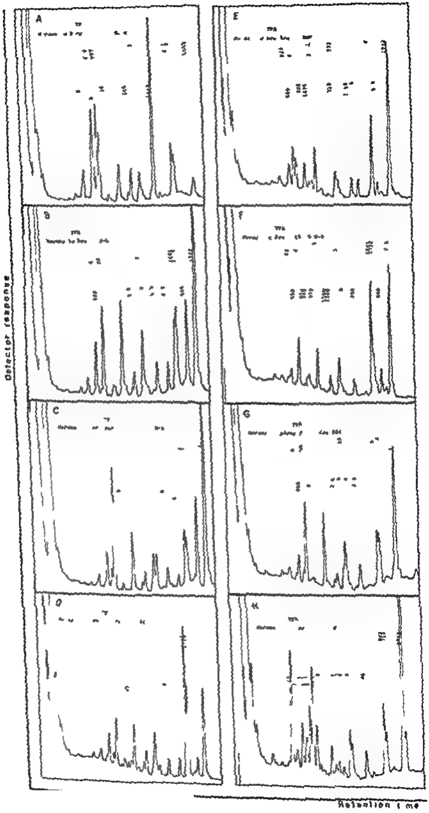
A
B
C
D
E
F
G
H
Detector response
Retention time

TABLE 2 *Relative Peak Height Da*

Peak retention time in seconds	M nonliquefaciens 4663/62	7784	3067/66	M bovis 10900	3, 4, 5
826				x	
907	x		x	x	x
1111	x	x	x	x	x
1333	x	x	x	x	x
1691			x	x	x
1714	x		x	x	
1942		x		x	x
660				[100]	
720				[100]	
780				[100]	
826	200	150	200	100	200
880	200	200	200	100	100
907	100	200	100	100	100
950				[100]	
1000	100	50	50	100	-
1075	200			[100]	
1111	100	100	100	100	100
1165				100	
1200				[100]	
1240	100	100	100	100	50
1310				[100]	
1333	100	100	100	100	100
1370	(100)	400	(250)	100	100
1490	100	100	100	100	100
1560				[100]	
1615	400	400	400	100	500
1691	150	(50)	100	100	50
1714	100	(100)	100	100	100
1840	50	(150)	100	100	(100)
1942	(50)	100	(100)	100	100

* TFA method see ref 19 for methodology and the text for explanation of the table and its use Chromatograms representative of all six species are shown in Fig 1

§ Tentative designation (20)

x Peaks used for reference in comparisons with *M bovis* 10900 N-a (reference peaks)

100 Approximate relative peak value indicating from 70 to 130 per cent of comparable peak in *M bovis* 10900 after correction for differences in relative height of reference peaks (see Results)

() The variation between parallels exceeds the ± 30 per cent range adopted as approximate reliability level for the present comparisons

M bovis The elution profiles of 10900 N-a and 4 N-b are shown in Figs 1F and 1B respectively Strains 3 4 and 5 were quite similar with respect to all peaks throughout the chromatograms and they are listed together in Table 2 The non-fimbriated or weakly fimbriated (N) and the strongly fimbriated (SC) varieties (Table 1) also differed very little, and the differences seen (not shown) are thought to be due to experimental variation When comparing strains 3, 4 and 5 (3 SC-c shown in ref 13) with 10900 N-a, it is found that the most significant difference is a 4–5 fold higher peak 1615 in the former strains One also notes a two-fold difference in peaks 826, 1240 and 1691

M kingii The elution profile of 4177/66

M kingii 4177/66	M kingii 9076/70	M. osloensis A1920	M. osloensis 5873	M phenylpyruvica 2863	M phenylpyruvica 17958	M urethralis§ WM6, WM20
				x	x	
	x		x	x	x	
x	x	x	x	x	x	x
x	x	x	x	x	x	
(x)	x	x	x	x	x	x
				x		x
		[2000]	[100]			
200	150	[400]	{200}			
		[1000]	[500]			
400	300	{200}	{200}	100	100	{200}
1000	1000	1000	200	200	200	500
150	100	200	100	100	100	50
		[1000]	[0]			
50	50	50	50	50	50	200
						[200]
100	100	100	100	100	100	100
		200	200			
		[200]	[100]			
200	200	50	50	100	200	(30)
		[500]	[250]	[200]		[200]
100	100	100	100	100	100	[illegible]
				(200)		
800	800	100	100	100	100	100
[200]		[200]				[500]
						50
50	50	75	75	50	50	(5–10)
(100)	100	100	100	100	100	100
	–	50	100	–	–	
20	20	<500>	<500>	100	50	100

– The peak value is very uncertain

[] The relative value is uncertain because the comparable peak in *M bovis* 10900 is hidden in the base line noise

{} The peak is probably heterogeneous, containing at least two substances This is judged from peak broadening and retention time deviation for peak maximum

<> Uncertain peak height value due to "off scale" chromatogram

SC-a is shown in Fig 1A (and that of 9076/70 SC-a *in ref 13)* Generally, all strains and varieties examined (Table 1) were quite similar in elution profile. Again, no difference was seen between non-fimbriated and fimbriated *variants*

M osloensis Of the two strains studied, A1920 is shown in Fig 1H *(the chromatogram of strain 5873 is shown in ref 13)* The peak labelled 950 is lacking *in strain* 5873 and the peaks 660 and 880 are far lower in the latter strain Otherwise the elution patterns of the two strains are rather similar *(Table 2)*

M phenylpyruvica The chromatogram of strain 2863 is shown in Fig 1G This strain and 17958 *(M polymorpha)* are again quite similar The differences amount to increases in the chromatographic areas corresponding to 1310 and 1370 sec retention

time in strain 2863 and a somewhat reduced 1912 peak in strain 17958 (Table 2)

M urethralis The GLC elution patterns of strains WM6 (shown in Fig 1E) and WM 20 were found to be so similar that they are grouped together in Table 2

Differences between Species

Careful analysis of all TFA elution profiles obtained indicated that comparison with *M bovis* 10900 was easiest in the sense that most species/strains studied showed the least difference from this one Consequently Table 2 was designed to facilitate this comparison (see above)

M nonliquefaciens Strain 4663/62 resembles *M bovis* 10900 in many respects The only major difference is a 4 fold higher peak 1615 in the former (this peak seems to be a characteristic of *M nonliquefaciens* and *M bovis* 3 4 and 5) Several peaks show a less significant twofold difference Strain 7784 reveals a similar strong resemblance to *M bovis* strains 3 4 and 5 The most significant difference here is a 4 fold higher peak 1370 in strain 7784 The 3067/66 chromatogram is also very similar to those of *M bovis* particularly strains 3 4 and 5

M kingii Typical of this species is the very high peak 1490 This is the major peak in the *M kingii* chromatogram whereas the other bacteria examined seem to have a well resolved minor peak in this area Also the 880 peak is very high in *M kingii* Increase in the latter region is also seen in *M osloensis* and *M urethralis* however The very low 1942 peak in *M kingii* is another very unusual and most significant feature With respect to the other peaks the differences are minor and not clearly distinguishable (except for peak 826)

M osloensis The most striking feature of this species is probably the very high 1942 peak although this peak with the exception of a few specimens has been the predominant one also in the elution patterns of *M nonliquefaciens* *M bovis* *M phenylpyrouvica* and *M urethralis* (but almost absent in *M kingii*) The 780 peak is also significantly increased showing well up in both chromatograms Further increased 880 and 1310 peaks seem characteristic The two strains studied differ somewhat so that all the peaks seen in the early part of the chromatogram in Fig 1H are not typical of all *M osloensis* strains

M phenylpyrouvica This species is again very similar to *M bovis* In fact the differences in elution profile (in the order of two-fold changes in relative peak heights) are so small as to be expected occasionally due to experimental variation

M urethralis A most demonstrable characteristic is the lack of a double peak in the chromatographic area around 1700 sec retention time It looks as if the 1691 peak is reduced to a shoulder on the front of peak 1714 (Fig 1E) The 880 peak is increased about 5 fold as compared to *M bovis* and a small peak is evident at 1560 sec (also *M kingii* and *M osloensis* have demonstrable peaks in this area of the chromatogram) The 500 per cent increase tabulated is put in brackets because there is no peak to compare it with in strain 10900 One cannot exclude however that a 5 fold smaller peak would disappear in the background noise in this case

In Fig 2 are shown TMS derivative chromatograms analysed on a different column of the four specimens *M bovis* 4 SCa *M nonliquefaciens* 7784 SCa and *M kingii* 4177/66 Na and SCa The *M bovis* and *M nonliquefaciens* elution profiles (Fig 2 A and B) are quite similar also by this derivatization method Again these two species are distinctly different from *M kingii* (Fig 2 C and D) Evidently N (non fimbriated) and the SC (fimbriated) varieties of the latter species have almost identical chromatograms

DISCUSSION

The aim of the present experiments was initially partly to investigate differences between fimbriate and non fimbriate bacteria *M* This was tested in *M nonliquefaciens* *M*

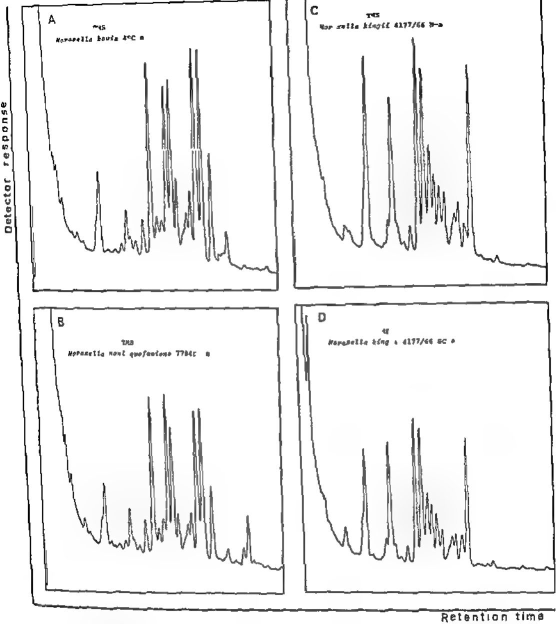

Fig 2 TMS elution profiles of moraxellae Lyophilized bacteria were methanolyzed trimethylsilylated and gas chromatographed (19

bovis and *M kingii* The early results did not point to any chromatographic difference between the varieties The small but distinct difference between *M nonliquefaciens* 7784 SC a and N a can be due to experimental variation Similarities as well as differences were found between the species that were consistent with previous taxonomic studies of these organisms (see below), and more strains and species were introduced (Table 1) to evaluate the GLC technique as a taxonomic tool The methodology employed has been discussed in the first paper of this series (19)

Presentation of the Data

Because of the similarities between the TFA chromatograms it is natural to compare region with region to find out whether the diagrams are identical in every detail or whether there are only a few common features In order to facilitate the comparison we decided to introduce retention times for the peaks detected and we chose to use a similar set of adjusted retention times in all chromatograms to be compared Between *Neisseria caviae* and *N ovis* chromatograms (13) there is a close resemblance This can also be seen on comparison of *N caviae* with *M bovis* (13) Because of this we felt we could use as reference the retention times determined (mean values from 9 chromatograms) for seven distinct peaks in *N ovis* (19)

Counterparts to most, if not all of the seven peaks mentioned were easy to find in all chromatograms compared (Fig 1, Table 2) although their relative height with respect to each other could differ over a wide range The relative position of these peaks along the abscissa (time axis) showed very little variation however When an individual peak was found to be broader than usual it was probably heterogeneous (see Table 2 legend) From the seven reference peaks in all chromatograms the retention times of intermediate peaks were calculated by interpolation Seemingly identical intermediate peaks will be found to have somewhat different retention times This points to inaccuracies of the method as well as to the obvious possibility of different chemical nature of the substances giving rise to apparently identical peaks Evidently the retention times introduced this way will not be correct *i e* equal to the actual emergence of the peaks in the experiments This is no valid objection to the method of comparison More serious is the danger of erroneous labelling of peaks with the same retention time implying that they are comparable chemically when they are not In fact we cannot say very much about the chemical identity of substances emerging with similar retention times in chromatograms of different species We tend however, to believe that identical features of different chromatograms point to chemical similarities although many peaks are probably heterogeneous and a trace component in one case may possibly represent the major entity in another (see also ref 19) The latter problem will be the subject of future experiments

The present paper is intended as a presentation of results that in our opinion strongly indicate how the GLC technique may be useful for the comparison of different microbes and thus serve taxonomic purposes

Taxonomic Evaluation

The results of TFA pattern analysis in the limited number of specimens studied in this initial investigation may be summarized as follows 1) Each *strain* gives a reproducible chromatogram There is no characteristic difference between fimbriated and non fimbriated variants of the same strain in the present system 2) Most often the chromatographic dissimilarities between strains of the same species are within or close to the experimental error 3) Occasionally there are clear *intraspecies* differences although the species allocation has been based on examination of nucleic acid composition and compatibility Such chromatographic deviations may reflect taxonomically important differences on the subspecies level Thus the three *M nonliquefaciens* strains examined and found to have slightly different elution patterns also differed slightly in terms of guanine + cytosine (G + C) average mole percentage of DNA (range 40 4 to 4? per cent refs 7 8) 4) When compared with *M bovis* the *M nonliquefaciens* and *M phenylpyruvica* strains show most similarity whereas *M kingii* shows the greatest deviation of chromatographic pattern *M osloensis* and *M urethralis* are intermediate in this respect

The appearance of the TMS elution curves confirms the results obtained by the TFA method These patterns were not analysed in such detail however

The taxonomy of the oxidase positive rod

shaped moraxellae has recently been extensively investigated and discussed particularly on the basis of tests for nucleic acid composition (G + C determination of DNA) and compatibility (genetic transformation and pulse RNA DNA hybridization). The GLC analysis presented gives supporting and supplementary information. Although improvement of the chromatographic system and the inclusion of a few more species/strains are needed for a complete discussion the chromatographic findings can be related to the previous knowledge of interspecies affinities in a meaningful way at the present stage.

Thus the pronounced chromatographic similarities between *M. nonliquefaciens* and *M. bovis* are consistent with the close relations between these "classical" moraxellae as revealed in genetic transformation (3) in interspecies pulse RNA DNA hybridization (6) and in metabolic studies (1).

M. kingii was found to have no affinities to other moraxellae in terms of genetic compatibility by transformation (17) or in pulse RNA DNA hybridization (6) in addition to the deviating cultural and biochemical characteristics of this species (17). The very distinct TFA and TMS elution profiles of *M. kingii* may be considered to weaken further its position as a member of genus *Moraxella* (18).

M. osloensis although forming a very distinct entity within genus *Moraxella* (1, 11) has some genetic affinities to *M. nonliquefaciens* and *M. bovis* (4, 5) and its G + C content of DNA is like that of *M. bovis* (8). The elution patterns obtained in GLC analysis are consistent with the assignment of this organism to genus *Moraxella* and also with the somewhat distant relations to the classical moraxellae mentioned.

The chromatographic similarity of *M. phenylpyrouvica* to *M. bovis* (and *M. nonliquefaciens*) constitutes an important information in favour of its present allocation to genus *Moraxella* (18). The genetic affinity of *M. phenylpyrouvica* to the other rod shaped moraxellae was only reported positive with respect to *M. osloensis* (12). However, it shows some genetic affinities to *N. catarrhalis* and *N. ovis*, as do also *M. nonliquefaciens*, *M. bovis* and *M. osloensis* (5, 12). It also has G + C contents of DNA in the range typical of genus *Moraxella* (8).

The similar elution profiles of the *M. phenylpyrouvica* neotype strain 2863 and the strain 17958 (= *Moraxella polymorpha* Flamm 1957 strain 1078/55) are consistent with a previous conclusion (12) that they belong to the same species. Their G + C contents of DNA differ only by about 0.5 per cent (8).

The *Moraxella* like *M. urethralis* (tentative designation) has as yet revealed no affinities to other moraxellae or neisseriae in genetic transformation (20) and has not been formally allocated to genus. The TFA pattern of this species is distinct but it shows considerably more similarities to unquestionable moraxellae than does *M. kingii*. The lack of the otherwise constant peak 1691 may, however, be of great taxonomic significance. A more general knowledge of the elution patterns obtained with this method in various other genera and families of microbes would be preferable for a complete discussion on this point.

On the basis of this study in a system of bacterial strains and species with largely well established taxonomy gas chromatography can be said to give reliable information for evaluation of relationships. When used together with other methods it may add significantly to the quality of classification, e.g. the allocation of species to genus. Still together with other methods it can be valuable also in species identification of an isolate. It may however in many cases be difficult to rely upon GLC analysis alone in such identification at least without improvements of the technical modifications used. The disturbing elements are close relations (also genetically) between some established species concepts together with minor intraspecies variations in elution profile. The latter differences could be useful as a means of subspecies differentiation, however.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

III Group Relations of Neisseriae and Moraxellae

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Fourteen strains of altogether eight species of neisseriae were 'fingerprinted' by gas liquid chromatographic (GLC) analysis of their trifluoroacetyl derivatized whole cell methanolysates The elution profiles were compared with each other and with those of previously analysed *Moraxella* species. The chromatograms of "false neisseriae" (*Neisseria catarrhalis, N ovis* and *N caviae*) and of cocci belonging to "true neisseriae" (*N cinerea, N flavescens, N meningitidis* and *N gonorrhoeae* examined) formed two relatively homogeneous groups distinctly different from each other The "false neisseriae" group showed a striking chromatographic resemblance to most species of the present genus *Moraxella* The *M kingii* profile, however, differing from other moraxellae, also differed from both groups of neisseriae The recently described rodshaped *Neisseria elongata* revealed as expected from nucleic acid compatibility tests, a chromatographic pattern almost completely identical to those of other 'true neisseriae' species The results therefore confirm previous conclusions on group relations in Neisseriaceae based on genetic affinities

Previous studies by means of genetic transformation, DNA base ratio determination and DNA-DNA and pulse RNA-DNA hybridization, have revealed generally consistent data on the nucleic acid affinities between species of moraxellae and neisseriae (see Discussion) The corresponding relations in gas liquid chromatographic (GLC) analyses have been discussed for moraxellae in the preceding paper of this series (13), in which the method of using TFA* derivatized whole cell methanolysates (16) proved to give reliable results when known genetic compatibilities were employed as parameters In this report the results of the same chromatographic method when applied to neisseriae, will be presented and related to the findings in moraxellae, and the group relations of these organisms will be commented upon

Received 16 iii 72

* TFA = Trifluoroacetyl

MATERIALS AND METHODS

Bacterial Strains

Fourteen strains representing eight species of neisseriae were included as specified in Table 1 The strains have previously been identified by conventional biochemical and cultural means, and their species allocation has to a large extent, been controlled by quantitative genetic transformation and DNA base ratio determination (see Table 1 for references)

TABLE 1 *Species and Strains of Neisseriae Examined by Gas Liquid Chromatography*§

Species	Strains	References
*N * catarrhalis*	Ne 11 13074/62	5 10, 16
*N * ovis*	199/55 37/59	5, 10, 16
*N * caviae*	ATCC 14659 (type strain) NCTC 10293	5 10
N cinerea	159/62	5 10
N flavescens	ATCC 13120 (type strain) NCTC 8263	5 10
N meningitidis	B8152/66 (serogroup A) M1 (serogroup B)	7, 16, 17
N gonorrhoeae	21319/70† 562/71†	
N elongata	M2	9

§ See ref 13 for material of rodshaped moraxellae examined

N * Temporarily named Neisseria see section of Discussion for other proposed genus names for these three species

† Recently isolated strains received from Dr A *Odegaard, Statens Institutt for Folkehelse, Oslo*

Methods

The bacterial strains were cultivated on the standard blood agar medium for 20 hr in a humid atmosphere (16) *Neisseria catarrhalis N ovis N caviae* and *N cinerea* were grown at 33° C The *N flavescens, N meningitidis, N gonorrhoeae* and *N elongata* cultures were transiently exposed to a small amount of CO_2 from the start of growth which took place at 37° C The two modes of incubation had no differential effect on the resulting elution profiles as shown in control experiments with *N meningitidis* B8152/66 (16) and with *N elongata* (not illustrated)

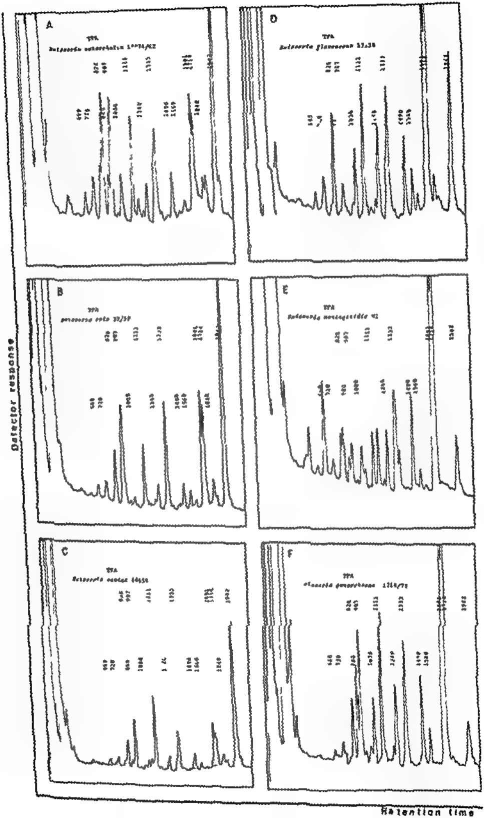
The methods of harvesting the cells and gas chromatography by the TFA method were as described (16) As in the preceding article (13) because their chemical identity has not been established the peaks in the chromatographic elution patterns are referred to by retention times in seconds

RESULTS

The TFA elution profiles of the *N catarrhalis, N ovis* and *N caviae* strains show a very close resemblance to each other (Fig 1 A, B and C) The chromatographic patterns of these "false neisseriae" (see Discussion) are characterized by seven dominating peaks (16), of which the peak at 1942 sec retention time is always the highest There is a constant height ratio between this peak and the distinctly lower double peak 1691 + 1714 Slight dissimilarities, like the apparent absence of peak 880 in *N ovis*, are seen but it remains to be clarified whether such chromatographic traits can be used for differentiation between the species (see ref 16 for chromatograms of *N catarrhalis* Ne 11 and *N ovis* 199/55) The "small peak pattern" seen just in front of peak 1111 in *N caviae* 14659 (Fig 1C) was not found in the *N caviae* 10293 elution profile, which was otherwise almost completely identical (not shown)

The three species *N flavescens, N meningitidis* and *N gonorrhoeae* ("true neisseriae", see Discussion) also reveal chromatographic peaks corresponding to the seven peaks selected as a basis for comparison of TFA elution profiles (13 16), as shown in Fig 1, D, E and F Relative heights of these and other peaks characterize the "true neisseriae" as a distinct group Thus the ratio between the double peak at 1691 + 1714 sec retention time and peak 1942 is turned around as compared to the group of "false neisseriae" the latter peak being about half

Fig 1 TFA chromatograms of neisseriae Lyophilized bacteria were methanolyzed trifluoroacetylated and gas chromatographed (16) The samples were from *different* harvests and were analysed at different times but under identical chromatographic conditions In the upper part of the chromatograms are given normalized retention times in seconds for seven selected peaks (16) and lower down retention times for intermediate peaks arrived at by interpolation See refs 13 and 16 for further explanation and comparable chromatograms of other strains and species


Detector response
Retention time
A
B
C
D
E
F
TPA

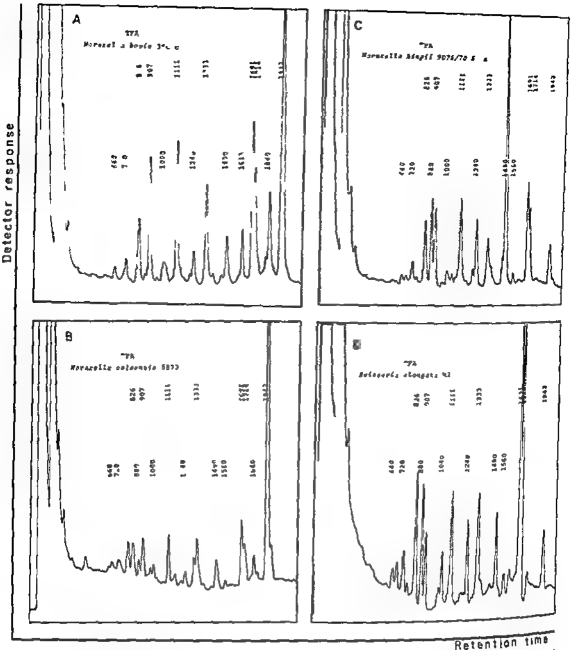


Fig 2 TFA gas chromatograms of three selected species of rodshaped moraxellae and of the rod shaped Neisseria elongata See Fig 1 and refs 13 and 16 for methodology explanation of the diagrams and comparable chromatograms of other *Moraxella* and *Neisseria* strains and species

of peak 1691 + 1714 heights in *N flavescens* and *N cinerea* (not shown), and only a fraction of them in *N meningitidis* and *N gonorrhoeae* Another common feature of the "true neisseriae" elution profiles is the considerable relative heights of peaks 1240 and 1490 The two strains of *N flavescens* and *N cinerea* had almost completely identical chromatograms, with a somewhat higher 1942 peak than the other species of the group The almost indistinguishable elution profiles of the two *N gonorrhoeae* strains were characterized by a clearly marked double peak 880 + 907 It is questionable

whether these characteristics are sufficiently specific for use in identification of "true neisseriae" The elution profile of *N meningitidis* M1 (Fig 1E) is identical to that of strain B8152/66 (shown in ref 16) in the 'late' (right) part of the chromatogram However, the high 660 peak and the distinct peak between retention times 1111 and 1240 (*i e*, at 1165 sec) in the M1 strain indicate that intraspecies chromatographic differences may sometimes be comparable to interspecies differences among these closely related organisms, as previously observed in moraxellae (13) It should be noted that the two strains of *N meningitidis* examined are of different serogroups (Table 1) In addition, the B8152/66 strain is distinguished by being asaccharolytic (7)

Careful analysis of the "false neisseriae" elution profiles and comparison with those of unquestionable moraxellae (13), of which *M bovis* and *M osloensis* are presented in Fig 2 (A and B), show that no general distinction can be made by this method between these two groups at present *M kingii*, on the other hand, appears clearly distinguishable from both groups of coccal organisms (Fig 2C) The *Moraxella* like organism tentatively called *M urethralis*, which revealed a characteristic lack of peak 1691 in the previous study (13) is also dissimilar from all neisseriae in this respect

The chromatogram of the rodshaped *Neisseria elongata* is shown in Fig 2D This elution profile is almost completely identical to that of *N gonorrhoeae*

DISCUSSION

Fatty acid composition (1, 3 19 21 22, 23) and to some extent the carbohydrate composition (22 23) of neisseriae have previously been investigated by gas chromatographic techniques The relevance of correlating chemical composition of bacterial surface structures with taxonomic relationship has been discussed in these reports In the present study of trifluoroacetylated whole cell methanolysates no efforts have been made to identify the peaks in the chromatographic patterns obtained The elution profiles are regarded only as easily obtainable "finger prints" which in recent investigations in our laboratories have proved valuable for taxonomic purposes (13, 16)

Studies on nucleic acid homologies by genetic transformation (5, 12) and DNA base ratio determination (10, 12, 20), and partly also by DNA-DNA hybridization (18) showed that the group of socalled "false neisseriae" (*N catarrhalis, N ovis* and *N caviae*) are distinctly interrelated, and, at the same time, without obvious compatibility with the relatively homogeneous group of "true neisseriae" (*N cinerea, N flavescens, N flava, N meningitidis, N gonorrhoeae* and other species) On the other hand, the former group reveals relatively close relations to some of the rodshaped moraxellae, particularly the species *M lacunata, M nonliquefaciens* and *M bovis* (2, 4, 6) As a consequence, *Henriksen* & *Bovre* (15) proposed that the "false neisseriae" should be included in genus *Moraxella* (in the family Neisseriaceae) The rationale of this proposal was later confirmed by means of interspecies pulse-RNA-DNA hybridization (8) Recently, however, *Catlin* (11) proposed a new genus name, *Branhamella*, for one of these species, *N catarrhalis*, although *Henriksen* and *Bovre* preferred not to create a new genus, because some moraxellae *e g*, *M bovis*, revealed higher genetic affinities to *N ovis* than did *N catarrhalis* (7) This "overlapping" compatibility being in favour of collecting all these coccal and rodshaped organisms in genus *Moraxella* was further substantiated in the hybridization studies (8) The present data from GLC analyses are corroborating, showing great dissimilarities in terms of elution profiles between the "false" and "true neisseriae each group being relatively homogeneous In this respect, the results are mostly consistent with those obtained by *Brooks et al* (3) in gas chromatographic studies of whole cultural extracts of neisseriae, to the extent that these

investigations can be compared At the same time, there are clear similarities between the chromatograms of false neisseriae and some rodshaped moraxellae Also the GLC method discloses 'overlapping' similarity

The elution pattern of *M kingii* is distinct from that of other moraxellae (13) The present study does not reveal clear resemblance to any of the neisseriae either This strengthens the notion based on nucleic acid in compatibility (8 14) that *M kingii* may only temporarily be placed in genus *Moraxella*

In the previous studies a new species of oxidase positive, rodshaped bacteria was discovered that showed high degree nucleic acid compatibility with the 'true neisseriae' but none with rodshaped moraxellae or 'false neisseriae' It was consequently named *Neisseria elongata* (8 9) Indistinguishable rod shaped organisms also in terms of heterospecific affinities in genetic tests have recently been found repeatedly (*K Bøvre, J E Fuglesang & S D Henriksen* unpublished) *N elongata* reveals as expected a very high gas chromatographic similarity to the coccal 'true neisseriae' e g to *N gonorrhoeae*

As a main conclusion the results of previous studies on group relations among moraxellae and neisseriae based on nucleic acid homologies and heterologies have been confirmed by the GLC analyses

ADDENDUM

After submitting this paper for publication we became aware of the very recent report by *Lambert et al* (Canad J Microbiol *17* 1491 1502 1971) on cellular fatty acids of nonpathogenic neisseriae To the extent that the two studies can be compared they are in agreement

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IDENTIFICATION OF PENICILLIN-STIMULATED STAPHYLOCOCCAL HAEMOLYSIN AS ALPHA-LYSIN

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Cultivation of S aureus in the presence of 0 03 IU benzyl penicillin stimulated the formation of a haemolysin which was suggested to be alpha lysin Its molecular weight was estimated to be 30000 by molecular sieve chromatography It was mainly active on rabbit erythrocytes and was neutralized by alphalysin antibody Furthermore it gave a reaction of identity in comparative immunoelectrophoresis with an arc which was shown to represent alpha lysin

In a previous paper (4) it was shown that penicillins in low concentration stimulated haemolysin production in some strains of Staphylococcus aureus The critical concentration ranged between 0 015 and 0 03 IU/ml benzyl penicillin A minor degree of stimulation was also obtained with chloramphenicol at low concentrations The phenomenon is typically demonstrated in antibiotic sensitivity tests with paper discs on sheep blood agar These results have been verified by other workers *Lorain* (6) observed stimulation by the penicillin group of antibiotics in 37 per cent of 126 investigated strains of Staphylococcus aureus and *Ritzerfeld et al* (8) in ⅔ of 118 strains

The dermonecrotic effect of staphylococci was also enhanced in rabbits which had received intramuscular injections of penicillin (5)

Four haemolysins alpha beta, gamma, and delta have been identified from S aureus (1, 7)

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The purpose of the present investigation was to identify the haemolysin, which is produced in increased amounts in the presence of low concentrations of benzyl penicillin

MATERIALS AND METHODS

Strain S aureus 12158 which is sensitive to benzyl penicillin was used in stimulation experiments S aureus Wood 46 was used for purification of *α lysin*

Antibiotic Benzyl penicillin (Kabi Sweden) was used for the stimulation of haemolysin production

Medium and cultivation For inoculation bacteria from 48 hour blood agar plate were suspended in saline The density of the bacteria was corrected to an extinction of 0 7 ± 0 05 in a Beckman C colorimeter (green filter)

0 5 ml of the bacterial suspension was inoculated into 10 ml of broth (0 3 per cent beef extract Difco 1 per cent peptone Difco 0 3 per cent sodium chloride) with or without the addition of 0 03 IU penicillin per ml 20 ml glass flasks with cotton plugs were used After 18 hours at 37° C on a rotary shaker the culture was centrifuged for 15 min at 4500 r p m

Assay of haemolytic effect Supernatants and fractions from gelfiltration experiments were tested with regard to haemolytic effect on rabbit human

and sheep red blood cells as described in a previous paper (1)

Gelfiltration of supernatants concentrated 10 times was performed on Sephadex G100 G50, and G25 (Pharmacia Fine Chemicals Uppsala, Sweden) The elution buffer was 0 02 M sodium phosphate containing ▮1 M NaCl pH 7 2 Elution was carried out at 4° C at 1 5–2 ml/cm²/hour and the effluent was collected in fractions of 5 ml

Preparative electrophoresis was performed on a vertical column (50 × 2 2 cm) packed with Sephadex G25 ▮ barbital buffer 0 05 M, pH 8 2 The electrophoresis was run towards the cathode at 500 V for 18 hours and the column was eluted at 1 5–2 ml/cm²/hour in 2 5 ml fractions

Purification of α lysin was performed in two steps 1) gel filtration on Sephadex G100 2) preparative electrophoresis

Antisera 1) Commercial Wood 46 antitoxin (Burroughs Wellcome et Co, lot nr RA 388 20000 units in 29 ml)

2) Antiserum to purified α lysin prepared from S aureus strain S6 as described earlier (3)

Immunoelectrophoresis was performed as described in an earlier paper (3) When haemolytic activity was to be localized the slides were overlaid with a 3 per cent suspension of washed erythrocytes in phosphate buffered saline (PBS) After 1 hour at 37° C the slides were rinsed in PBS

Neutralization 0 5 ml toxin was mixed with an equal volume of serum inactivated for 30 min at 56° C After incubation at 37° C for 1 hour the mixture was back titrated for non neutralized haemolytic activity

Trypsin inactivation 0 2 ml of a trypsin solution —0 1 mg/ml in PBS (Worthington Biochemical Corp N J, U S A, 3 x cryst) was added to 1 8 ml of sample pH 7 2 After 30 min at 37° C, 0 1 ml of soybean trypsin inhibitor—0 1 mg/ml in PBS (Sigma Chem Corp, Miss, U S A 2 x cryst) was added to 0 4 ml of the haemolysin trypsin mixture

EXPERIMENTS AND RESULTS

Gelfiltration Experiments for Approximation of Molecular Weight

To estimate the molecular weight of penicillin induced haemolysin supernatants from cultures containing 0 03 IU benzyl penicillin or no penicillin were gelfiltered in parallel at 4° C through Sephadex G100 All fractions were analysed for haemolysis of human, rabbit and sheep erythrocytes As shown in Fig 1 the haemolytic activity in both cultures was separated into two peaks, one eluting with the void volume, mainly haemolysing human erythrocytes, and one at 45–65 per cent of the bed volume active on rabbit erythrocytes This distribution is typical for delta and alpha lysin, respectively (2) Sheep erythrocytes were only slightly affected by first peak fractions

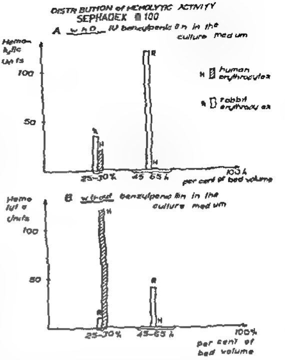

Fig 1 Distribution of haemolytic activity on human and rabbit erythrocytes after gel filtration through Sephadex G100 of penicillin stimulated and non stimulated culture supernatants of S aureus 12158

It is of interest that material from the penicillin containing culture contained significantly more haemolysin active against rabbit erythrocytes localized in the second peak than material from the penicillin-free culture Furthermore, penicillin caused a decrease in large molecular weight haemolysin active on human cells

When gelfiltered through Sephadex G25 the penicillin stimulated haemolysin eluted with the void volume as shown in Fig 2 On Sephadex G50 it appeared closely after the void volume at 30–35 per cent of the bed volume, indicating a molecular weight of approx 30000, which agrees with α-lysin

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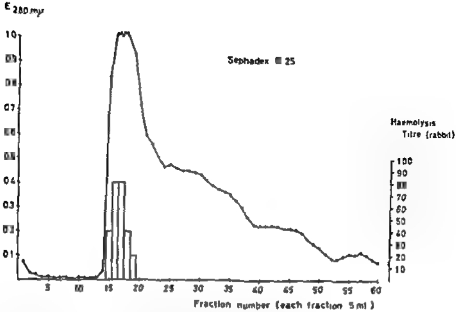

Fig 2 Distribution of haemolysin active on rabbit erythrocytes after gel filtration through Sephadex G25 of penicillin-stimulated culture supernatant of S. aureus 12158

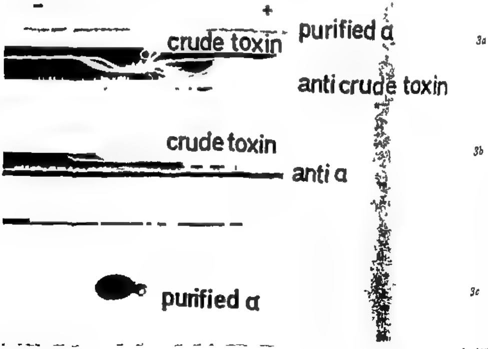

Fig 3a Interaction of purified α lysin from non stimulated S aureus Wood 46 *with the* crude toxin-crude antibody toxin system

Fig 3b The same crude toxin developed with an α lysin antibody prepared from purified α lysin of strain S6

Fig 3c Localization of purified α lysin after electrophoresis The slide was run in parallel with those shown in Fig 3a and 3b

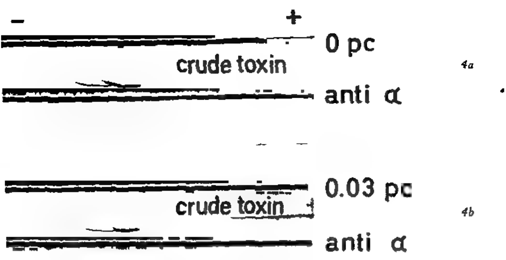

Fig 4a and b Interaction of non stimulated and penicillin stimulated culture supernatants of S aureus 12158 with the crude toxin—anti-α system

Immunoelectrophoretic Identification of Penicillin Induced Haemolysin

For further characterization, the induced haemolysin was studied in immunoelectro phoresis In a first experimental model crude toxin from strain Wood 46 was developed with a Wood 46 crude antitoxin (Fig 3a) as well as antiserum prepared from purified α lysin of S aureus strain S6 (Fig 3b) This is a well characterized strain which produces α and γ lysin but not β lysin In the anti α-serum two arcs were present The precipitation arc representing α lysin was identified with a purified α lysin fraction from Wood 46 (Fig 3a) The localization of purified toxin after electrophoresis was also visualized with an overlay of rabbit erythrocytes (Fig 3c)

In another series of comparative electro phoresis (Fig 4a and 4b) non concentrated supernatant from one culture grown with and one without 0 03 IU penicillin was allowed to develop against the crude toxin anti-α system A reaction of identity was obtained between penicillin activated supernatant and the α line (Fig 4b) No such reaction could be recognized in the case of supernatant without penicillin (Fig 4a)

Neutralization

As shown in Table 1, the α lysin antibody also totally neutralized all haemolytic activity Pre immuneserum from the same rabbit was inactive in this respect

Inactivation by Heat and Trypsin

All haemolytic activity given by cultures grown in the presence of 0 03 IU penicillin per ml was destroyed by heating at 100° C for 1 min or exposure to trypsin at 37° C for 30 min

DISCUSSION

In routine antibiotic sensitivity test with penicillin discs, the haemolytic rings surrounding the inhibition zones of staphylococcal growth indicate a stimulation of haemolysin pro duction This has been verified in broth cultures containing penicillin

The molecular weight of this haemolysin was approximated to 30000 by molecular sieve chromatography Gel filtered fractions were active on rabbit erythrocytes, but not on human or sheep red cells Molecular weight, heat instability as well as species spectrum is consistent with alpha lysin (1)

TABLE 1 *Neutralization with α Anti Toxin*

Culture grown with	Haemolysis rabbit erythrocytes titre	Neutr with preimmuneserum titre	Neutr with α anti toxin
0 1U pc/ml	1/4	<1/4	<1/4
0 3 IU pc/ml	1/64	1/64	<1/4

Delta lysin, on the other hand, will elute in the void volume on Sephadex G100, indicating a large molecular weight (2) Neither is it affected by heating to 100° C for 1 min

The species spectrum in gel filtered fractions excludes beta lysin which is identified by the haemolysis of sheep or ox erythrocytes enhanced at 4° C It does not haemolyse rabbit erythrocytes On the other hand, the initial observation was made on sheep blood agar, though it was not intensified by refrigeration The activity on sheep blood might be explained by a simultaneous liberation of synergistically acting toxin It should also be pointed out that strain S6 dos not produce beta lysin

It is probably not gamma lysin as this is unique in being inhibited by agar (7)

It is also unlikely that the phenomenon can be explained by the formation of a new haemolysin specifically induced by penicillins Such a haemolysin would not have been neutralized by the alpha lysin antibody used as this was prepared from non induced strain S6 material

In immunoelectrophoretic analysis a reaction of identity developed between induced culture supernatant and an arc which was identified as alpha lysin

In conclusion most findings suggest alpha lysin as the liberated substance This is in agreement with the observation of *Rit erfeld et al* (8) that rings of haemolysin surrounding penicillin discs were obtained only with alpha lysin producers

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STUDIES ON INCREASED TURNOVER OF BRAIN MONO-AMINES INDUCED BY EXPERIMENTAL HERPES SIMPLEX INFECTION

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Intracerebral infection of mice with different herpes simplex virus (HSV) strains caused raised dopamine and 5 hydroxytryptamine turnover, reflected in increased brain concentrations of the acid metabolites of the amines i e homovanillic acid (HVA) and 5 hydroxyindole acetic acid (5 HIAA) Inhibitors of virus multiplication (actinomycin D, mitomycin C and iododeoxyuridine) and different variants of HSV were used to study whether the rise in mono-amine metabolism was an event dependent upon the direct action of the virus or viral metabolites on pathways of mono-amine synthesis The results obtained were not compatible with such a hypothesis The histopathological study of the distribution of the neuronal lesions indicated that the lower brain stem from which most of the mono aminergic neurons originate was hardly affected at all The degree of severity of the neuronal lesions in relation to the HVA and 5 HIAA concentrations found in infected brains was studied for 13 different HSV variants The more extensive and frequent the lesions the higher were the levels of the acids encountered It was assumed that impaired functions of postsynaptic cells or altered receptor functions possibly affecting regulatory feed back mechanisms could be responsible for the increased mono amine turnover

In mice intracerebrally infected with virus an increased neuronal activity, characterized by raised release and synthesis of brain catecholamines and 5 hydroxytryptamine (5 HT) was observed (12 13) Studies on the relationship between release of amine in the infected brain and virus multiplication as well as the dose response relationship between amount of virus inoculated and the concentrations of the acid metabolites, homo vanillic acid (HVA) and 5 hydroxyindole acetic acid (5 HIAA), observed, indicated that there existed causality between the virus infection and the rise in the mono amine turnover Inhibitors of dopamine (DA) and 5 HT synthesis (1) were able to eliminate the virus induced effects on the mono amine metabolism without inhibiting the virus multiplication suggesting that most of the adrenergic neurons in the infected brains were functionally intact (12)

In the present investigation, substances known to interfere with the herpes simplex virus (HSV) multiplication were used for the study of the relationship between response of the mono amine metabolism and

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inhibition of the virus multiplication Variants of HSV with different biological characteristics were used to discover whether genetic differences of the variants were also reflected in their capacity to affect the mono amine metabolism of the infected brains Finally, the differences in the pattern of mono-amine metabolism with different HSV strains were studied against the background of the histopathological picture

MATERIAL AND METHODS

Virus

A type 1 strain (St 2 Gbg 12) of HSV, propagated by several intracerebral passages in mice and some different variants originating from HSV strains isolated from 10 clinical cases of herpetic infections were studied Among the latter strains 4 were characterized as type 1 strains and were isolated from cases with oral herpetic lesions Six were registered as type 2 strains, and were isolated from cases with genital or neonatal infections with HSV By plaquing the strains on green monkey kidney (GMK) cell cultures variants differing with regard to size of plaques produced were isolated Some characteristics of these variants are described under results All virus suspensions were stored at —90°

Cell Culture

A green monkey kidney (GMK) cell line was cultured at 37°C in 5 cm wide plastic petri dishes using Eagle s medium (MEM) supplemented with calf serum and antibiotics For plaquing of the virus the same medium as described above but containing in addition 1 per cent methylcellulose or agar was used The cultures were incubated at 37°C and the plaques were registered on the fourth day after virus inoculation Titres were expressed in plaque forming units (pfu) per ml Means of 3 titrations are given in text and tables

Cloning Procedure

Dilutions of the original HSV strains giving single or a few plaques per culture were prepared Cells from a plaque defined as small or large were collected transferred to a culture tube and a virus suspension was produced This suspension was seeded on new culture dishes the plaques were examined and the procedure repeated At least 3 but sometimes up to 10 consecutive clonings were performed to ascertain the isolation of a particular HSV variant Only variants which seemed to be stable with regard to plaque morphology were used

Mice

Swiss albino mice, 22 23 days old of our own laboratory breed were used For studies of monoamines and histopathology the animals were anaesthetized with ether and inoculated intracerebrally with the virus 0 02 ml containing 60 LD_{50} doses per mouse The brains were harvested on the fourth day after the virus inoculation Titration of virus infectivity was performed on groups of mice 6 animals per group The virus suspensions were serially 10 fold diluted and each dilution was inoculated intracerebrally into a group of mice 0 02 ml per mouse The animals were examined after 14 days and titres in log units of LD_{50} were calculated according to *Reed & Muench* (14)

Biochemistry

Determinations of homovanillic acid (HVA) were made spectrophotofluorometrically according to *Andén et al* (1 10) Assays of 5 hydroxy indoleacetic acid (5 HIAA) were made according to *Jonsson & Lewander* (9) The accuracy of the determinations has been reported previously (12) If not otherwise stated the results are expressed as means of 3–10 determinations with standard errors of the means

Histopathology

Heads of mice were fixed in Heidenhain s Susa overnight before opening the skulls and removing the brains Sections 10 µ thick were stained with Giemsa and with luxol fast blue cresyl violet Both hemispheres were examined frontoparietal and occipitotemporal cortex olfactory bulbs hippocampal region septal areas neostriatum thalamic nuclei, substantia nigra cerebellum and pontine nuclei The neuronal lesions were graded according to a four graded scale (0 implying that no lesions were observed 1 that a few degenerating nerve cells in one or two of the regions were found 2 that moderate neuronal degeneration or necrosis in a few of the regions 3 that extensive and frequent lesions were seen) All sections were read under coded numbers

Virus Inhibitors

Three different substances known to inhibit the multiplication of HSV were tested Actinomycin D (Lyovac® Merck Sharp and Dohme U S A) was used in a concentration of 0 1 µg/mouse Mitomycin C (Sigma Chemical Co U S A) in 0 6 µg/mouse and iododeoxyuridine (Iduridin® Ferring Sweden) in amounts of 30 µg/0 02 ml and mouse The inhibitors were mixed with the virus suspensions and inoculated intracerebrally simultaneously with the virus

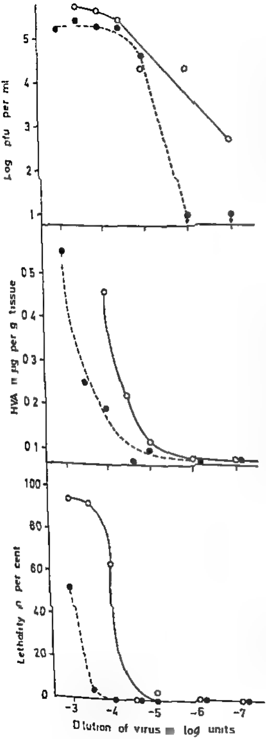

Fig 1 Effect of actinomycin D, 0 1 µg/mouse, in herpes simplex infected mice Results obtained with actinomycin D treated animals are indicated by dashed curves and filled symbols untreated controls by solid curves and open symbols Concentration of infective virus in log pfu/ml of a 20 per cent suspension of brain tissue, amount of homovanillic acid (HVA) in µg/g brain tissue or lethality in per cent are plotted against the concentration of virus inoculated in log units of dilution of virus suspension used The 25 animals in each group were inoculated intracerebrally with 0 02 ml of a virus suspension to which actinomycin D was added Four days after the inoculation the mortality was registered and the brains were harvested for assay

RESULTS

Influence on HVA Content by Inhibition of Virus Multiplication

In HSV infected mice the increased brain concentrations of HVA are as a rule 6–7 times above the levels encountered in normal uninfected mice (12) The influence of a suppressed virus infection on the formation of HVA was studied by inhibiting the virus infection with actinomycin D, mitomycin C or iododeoxyridine The virus suspension (St 2 Gbg 12) was diluted serially in half $\log_{10}$ dilution steps The drugs were added to the virus dilutions giving the final concentration of the drug mentioned under methods Twentyfive mice were inoculated with each mixture of the virus dilution and the drug As controls groups of 25 mice were inoculated with the virus dilutions alone or with the drug alone, concentrations and volumes all corresponding to those of the above mixtures of the virus and drug Death rates were registered and 4 days after the inoculation the surviving mice were killed and the brains were harvested and analysed for contents of HVA and infective virus

Under the experimental conditions ... the drugs had no noticeable effect ... concentrations of HVA in uninfected ... mals Fig 1 summarizes only the ... obtained with actinomycin D ... obtained with the other two drugs ... practically identical with ...

TABLE 1 *Designations and Some Characteristics of 13 HSV Mutants and Concentrations of HVA and 5 HIAA as well as Neuronal Lesions in Brains of Mice Infected with These Mutants*

Mutant**	Source	No of clonings	No pfu per LD_{50}	µg per g tissue of HVA	µg per g tissue of 5 HIAA	Score of neural lesions*
MA small	genital	3	6	0.28 ± 0.01	0.47 ± 0.05	2
CB small	neonatal	3	2	0.14 ± 0.01	0.36 ± 0.03	1
FIB small	neonatal	7	2	0.40 ± 0.03	0.68 ± 0.05	2
GF small	oral	3	4	0.20 ± 0.02	0.74 ± 0.06	3
KJ large 502	oral	6	1	0.27 ± 0.03	0.38 ± 0.04	2
KJ large 503	oral	6	0.2	0.26 ± 0.04	0.39 ± 0.03	2
HJ small	oral	3	3	0.31 ± 0.02	0.62 ± 0.03	2
IL small	genital	7	2	0.25 ± 0.02	0.47 ± 0.01	0
IL large	genital	10	100	0.20 ± 0.01	0.31 ± 0.02	0
TN small	neonatal	3	10	0.19 ± 0.05	0.41 ± 0.05	1
AO small	genital	3	1	0.08 ± 0.01	0.31 ± 0.03	0
MS large	oral	3	3	0.49 ± 0.11	0.52 ± 0.13	3
MS small	oral	7	2	0.44 ± 0.06	0.61 ± 0.03	2
Uninfected***				0.08 ± 0.01	0.32 ± 0.01	

* For grading of scores see Methods
** "small" or "large" indicate the plaque character
*** c f Lycke E, Modigh, K & Roos B E[19]

for actinomycin D Actinomycin D inhibited the virus multiplication, reduced the brain concentrations of HVA and decreased the death rates with approximately the same efficiency

Concentrations of HVA and 5-HIAA in Brains of Mice Inoculated with Different HSV Mutants and Histology of Brains Studied

Stock suspensions of 13 HSV plaque variants were studied Sixty to 120 mice were inoculated per variant, using the intracerebral route and a dose of 60 LD_{50} per mouse Brains harvested on the fourth day after inoculation were examined histologically and concentrations of HVA and 5-HIAA were determined Results obtained are summarized in Table 1

The concentrations of HVA and 5-HIAA of the brains differed markedly with regard to the HSV strain used for infection Genetic differences associated with type of virus, plaque morphology and number of plaques, were apparently of no importance for this variation Nor seemed the variation in HVA and 5 HIAA concentrations to depend upon

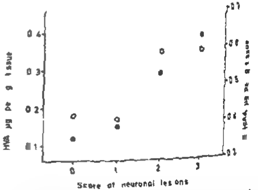

Fig 2 Relationship between degree of severity of neuronal lesions and concentrations of homovanillic acid (HVA) and 5 hydroxyindoleacetic acid (5 HIAA) in mice intracerebrally inoculated with different strains of herpes simplex virus Concentration of HVA (left ordinate and open symbols) and 5-HIAA (right ordinate and filled symbols) are plotted against scores of neuronal lesions (see methods) All strains were inoculated in 60 LD_{50} doses per mouse and brains were harvested on the fourth day after the virus inoculation

differences between the strains in their virulence to mice There was no correlation to death rates or to the pfu/LD_{50} ratios observed for the individual strains

The histopathological study revealed that the regions most commonly affected were the olfactory bulbs the pyramidal band and fascia dentata and the thalamic nuclei Only occasionally were lesions seen in the septal area, pontine nuclei, substantia nigra and cerebellum while the cerebral cortex and neostriatum were, generally, totally unaffected In order to register the degree of severity of the neuronal lesions caused by the different strains, scores graded from 0–3 were estimated as described under methods When the calculated means of the HVA and 5-HIAA concentrations were plotted against these scores a relationship was detectable (Fig 2) Thus, the more pronounced the neuronal lesions the higher were the concentrations of the acid amine metabolites in the brains

DISCUSSION

Experimentally induced viral encephalitis will in mice cause increased release and synthesis of DA and 5 HT, reflected in rises in brain concentrations of the acid metabolites HVA and 5 HIAA (12 13) There are several ways by which viral infections may cause dysfunctions in mono aminergic neurones and the pathogenesis of such dysfunctions may even be different for various virus infections

In the *herpes simplex* encephalitis the histopathological changes are preferably located in the pyramidal bands, the areas around the ventricles and in the olfactory bulbs while the lower brain stem is usually unaffected In agreement with these findings, the mono aminergic neurones, starting mainly from the brain stem, are according to histofluorometrical observations (6) essentially intact also in encephalitic animals Consequently inhibitors blocking the pathways of catecholamine and 5 HT synthesis can effectively inhibit the pathological rise in amine formation induced by the virus infection (12)

At first these observations were interpreted as suggestive of an interference of viral metabolites in the production of the amine-synthezing enzymes, leading to an uncontrolled formation of amines Such a hypothesis seemed less plausible, however, when it was found that quite unrelated virus infections, cause by DNA as well as RNA-viruses could provoke a similar increase in release of amines (12) Nor did the observations reported in the present study support this assumption Although the capacity to induce a pathological rise in mono amine turnover could vary between individual strains of HSV, the variation did not seem to be associated with genetic characters expressed in type, plaque-morphology or difference in virulence for mice

Inhibition of raised DA turnover in HSV infected mice could be obtained with substances like actinomycin D capable of blocking the HSV multiplication These observations stressed the causality between the viral infection and the pathological rise in formation of HVA Sometimes relatively high concentrations of infective virus were observed in brains revealing a normal content of HVA Such differences might well be explained as being of a quantitative rather than qualitative nature However, they did not favour the assumption that raised turnover of amine would be the result of a direct interference of virions or of a viral metabolite with the DA synthesis The most plausible explanation seemed to be that there existed an intermediate determinative step between the virus multiplication and the pathological increase of HVA induced by the infection

Under the experimental conditions applied the drugs had no demonstrable influence on HVA formation in normal uninfected mice As the effect of the virus inhibiting substances on the amine turnover somehow had to be mediated by the action of the drugs against the virus infection, it was reasonable to assume that their effect on DA turnover

might be secondary to a reduction or a delay in the development of the cytopathic changes in the HSV infected brains This suggestion was supported by the findings of a certain correlation between the concentrations of HVA and 5 HIAA determined versus the extent and severity of the neuronal lesions observed A similar correlation was noted when the development of the neuronal destruction was arrested by incubation of HSV infected mice at increased environmental temperature (11)

Provided the mono-aminergic neurones of the virus infected animals are intact as regards their synthesizing capacity, which seems to be the case, the reported findings draw attention to the receptor functions Destruction of postsynaptic effector cells and the receptors or modifications of the receptors might affect regulatory feed back mechanisms as would *e g* adrenergic receptor-blocking agents (3, 5)

The reported observations should be seen as a part of a study aimed at a testing of the hypothesis that an infectious event might be the initial step in the pathogenesis of Parkinsons disease A background for such a theory has already been presented (12), but ought to be supplemented by reference to the discussion of the possible importance of slow virus infections for the development of chronic neural disease in man—see *e g Gibbs et al* 1968, *Gibbs & Gajdusek* 1969 *Brody et al* 1970 In the study of the interaction of virus infection with mono amine metabolism in mice, HSV was chosen due to the pronounced increase in the release of amine and the accumulation of acids The use of the HSV infection involves obvious disadvantages however mainly the low resistance of small laboratory animals to the infection which, in turn, will limit the observation periods Studies with avirulent and attenuated virus strains are therefore in progress and will be reported on later

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We are indebted to Dr *K Kristensson*, who performed the histopathological examinations and to Miss *Birgitta Thoreson* Mrs *Gun Andersson* Mrs *Ann Britt Forsstrom* Miss *Anna Carin Boson* and Mrs *Gun Lundblad* for skillful technical assistance

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CORRELATION BETWEEN THE OCCURRENCE OF PROTEIN A AND SOME OTHER PROPERTIES IN *STAPHYLOCOCCUS AUREUS*

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The prevalence of protein A producing strains of *S aureus* was found to vary An increase in the proportion of weak protein A producers during the past decade was found to coincide with a shift in dominant phage types in Denmark Within phage groups I and II the ability to form large amounts of protein A was shown to be correlated to a deficient haemolysin production This correlation was also found for group III strains susceptible to phage 75 A Among group III strains susceptible to phages within the complex 83A 84 85 6557, 89 two subgroups were remarkable The strains which were resistant to neomycin (antibiogram PSTEN) produced large amounts of protein A whereas those which were resistant to methicillin (antibiograms PSTEM and PSTM) produced little or no protein A Analysis of the present material revealed no demonstrable or suspected pathogenic properties of protein A

The frequency of protein A production in staphylococcal strains has been studied by *Jensen* (8), *Lind* (13), *Forsgren* (6) and *Kronvall et al* (12) Protein A was detected in 75 to 99 per cent of the coagulase positive strains of *Staphylococcus aureus (S aureus)*, whereas among coagulase-negative strains only *Forsgren* (6) has demonstrated protein A, and then only in a small proportion (2 per cent) *Kronvall et al* (12) found no correlation between the absence or presence of protein A and other characteristics of *S aureus*

Received 6 iv 72

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The present material comprises 457 coagulase positive strains of *S aureus* The ability to form protein A was correlated to the following properties phage type, production of extracellular lipase resistance to mercury resistance to antibiotics and the production of α-, β and δ haemolysins

Preliminary results of this study have been published elsewhere (14)

MATERIALS AND METHODS

Bacterial strains S aureus strains from three different materials were screened for their capacity to form protein A The strains originated from all over Denmark and were received from the Department of Hospital Infections, Statens Seruminstitut (Head *K Rosendal* M D) The strains isolated in 1961/62 and 1965/66 (see Fig 3) were chosen

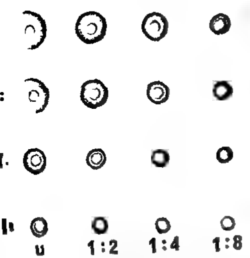

Fig 1 Quantitative prote n A determination by a modif ed Mancini technique (15) *Hori ontal* Di lution of protein A antigen (crude preparation) *U* Undiluted *Vertical* Four strains are tested, one for each of the groups designated by FAT as follows + + + + = A, + + + − B, + + = C and + D Group O gave no precipitate

n random from the routine specimens whereas the strains used in the present comparative study were selected in the following way one third orig nated from strains exhibiting strong protein A reactivity in the 1965/66 material (blank columns marked + + + and + + + + in Fig 3) another third were strains originating from cases of bacteriaemia (11) isolated in the same years and k ndly supplied by Dr *Rosendal* who also provided the last third of the material which consisted of bacteriaemia strains with known antibiogram (see below) All strains were coagulase positive and fermented mannitol

Phage type production of extracellular lipase and resistance to mercury Information about these properties was kindly supplied by Dr *Rosendal*

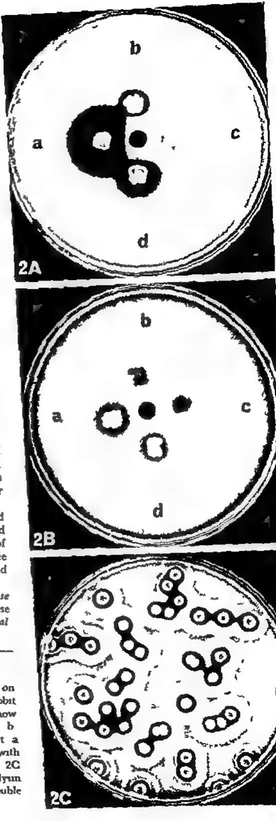

Fig 2 Detection of α β and δ haemolysins on agar plates incorporating erythrocytes from rabbit (A) horse (B) and sheep (C) 2A and 2B show examination of four strains (a Wood 46, b Cowan 1 c 4972 d 20036) inoculated at a distance of 7 mm from a disc impregnated with anti-α-staphylolysin (international standard) 2C shows 48-hour-old single colonies of a β haemolysin producing strain Note the characteristic double zone

Phage typing was performed by the method of *Blair & Williams* (1) with the phages 84, 85 89 and the experimental phage 6557 (2) added to the set The term non identifiable implies that the strain could not be referred to a single sero logical group, and non typable that it was not typable with this standard set of phages The methods used for demonstration of extracellular diffusible lipase (Tween 80 reaction) and resistance to mercury have been described in (9) and (10), respectively

Fluorescent antibody test (FAT) The binding of fluorescein isothiocyanate (FITC) labelled γG globulin to protein A offers a means of simple screening of strains for their capacity to form this antigen (13 16) Each strain was tested at least twice in the FAT using FITC labelled normal globulins from rabbit man or swine On the basis of the protein A reactivity in FAT the strains could be divided into five groups which were graded O (negative) and from + to ++++ A ++++ reaction corresponds to that found for the strain Cowan type 1 (NCTC 8530), which is known to be rich in protein A *(8, 12, 13 16)* The grouping by this method closely corresponded to that obtained for 60 representative strains, in which the protein A production was measured quantitatively by means of a modified Mancini technique (Fig 1) *(15)*

Resistance to antibiotics The pre diffusion method described by *Thomsen* was used *(17, 18)* The following antibiotics were included penicillin (P), streptomycin (S), tetracyclines (T), erythromycin (E) neomycin (or kanamycin) (N) and methicillin (M) For the determination of resistance to methicillin the plates were incubated at 30° C instead of 36° C (7) For each strain a symbol of capital letter(s) indicates the antibiotic(s) to which this strain was resistant or showed a significantly decreased sensitivity (the antibiogram)

α β and δ haemolysins As recommended by *Elek* (5) the production of haemolysins was determined by means of their action on erythrocytes incorporated in agar plates Erythrocytes from *three species (rabbit sheep and horse) were used* and typical zones of haemolysis are shown in Figs 2A 2B and 2C α haemolysin acts on erythrocytes from rabbit and sheep the border line of the zone of haemolysis is diffuse and the haemolysis is inhibited by anti α staphylolysin (international standard obtained from the Department of Biological Standardization, Statens Seruminstitut) (Fig 2A) β haemolysin acts only on sheep erythrocytes giving a characteristic double zone (Fig 2C) δ haemolysin forms a narrow and well defined zone of haemolysis on plates with erythrocytes from all three species (Fig 2B)

RESULTS

Screening for protein A production Fig 3 shows the distribution of protein A reactivity in the three different materials of *S aureus* The hatched columns represent 115 strains isolated during the years 1961–62 The distribution within the five groups of protein A reactivity is quite even About 10 per cent of the strains produce an amount equivalent to that found for Cowan type 1 (++++ reaction) Four years later (the blank columns) a material consisting of 706 strains showed a distribution in which the percentage of weak producers had increased considerably The black columns show the protein A reactivity of the 457 strains used in this study, they were selected as described under Materials and Methods

The strains which in the following are designated protein A positive (pA pos) correspond to the groups marked +++ and ++++ on the figure, protein A negative (pA neg) correspond to O and +, the larger intermediate group (++) is designated protein A variable

Correlation to phage type Protein A was formed by strains of phage groups I *II and III (Table 1)*

Within group I susceptibility to phage 29 was associated with a high protein A con

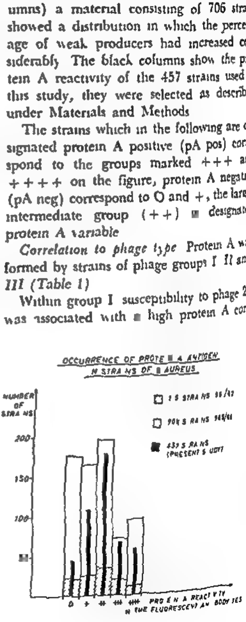

Fig 3

tent No pA neg strains were found among strains susceptible to the phage complex 52, 52A 80 81 and about 60 per cent (21/35) of these strains were pA pos

TABLE 1 *Distribution of 455 Strains of* S aureus *According to Phage Type and Protein A Reactivity in the Fluorescent Antibody Test*

Phage type	Fluorescent antibody test Positive	Variable	Negative	Total
Group I	26	30	3	59
Group II	7	11	12	30
Group III	77	118	123	318
Non identifiable	9	10	8	27
Non typable	5	9	7	21
Total	124	178	153	455

Within phage group II the pA neg strains were all susceptible to phage 71 The pA pos strains were susceptible to phages within the complex 3A 3B 3C 55 two of these strains were in addition susceptible to phage 71

Within group III the ability to form protein A was a common characteristic of two subgroups One consisted of strains susceptible to phage 75A and the other was found among strains lysed by the phage complex 83A 84 85 6557 89

The distribution of protein A reactive strains within the phage groups marked *non identifiable* and *non typable* was quite even

Correlation to Hg resistance and Tween 80 reaction Protein A production was not correlated to these two properties They were however associated with phage type and with resistance to penicillin as described in other reports (*e g* 10 3)

Correlation to production of haemolysins The percentage of strains producing α haemolysin was comparatively low (83 per cent for the whole material 63 per cent within phage group I and 87 per cent within phage group III) Strains susceptible to the phages 29 and 75A was found seldom to produce α haemolysin (4/21 and 2/20 respectively), in the material selected on basis of reactivity in FAT a certain accumulation of these strains occurred thus contributing to low average values

The haemolysin pattern αδ is predominant, however, strains which produce only α haemolysin, or which do not produce haemolysins at all are remarkably common Table 2 shows the distribution according to protein A reactivity and to haemolysin pattern (only those patterns which are related to human pathogens (5) are included) Among the pA neg strains the pattern αδ is predominant and those strains that do not produce δ haemolysin comprise only 7 per cent (10/153) of the group In contrast the distribution among pA pos strains reveals 32 per cent (39/123) with the pattern αδ and 59 per cent (72/123) which do not produce δ haemolysin 24 strains (19 per cent) of the latter do not produce haemolysins at all The distribution in the intermediate, protein A variable group resembles that found for pA neg strains

TABLE 2 *Distribution of 448 Strains of* S aureus *According to Protein A Reactivity in the Fluorescent Antibody Test and Haemolysin Pattern*

Haemolysin pattern	Fluorescent antibody test Positive	Variable	Negative	Total
αβδ	2	2	8	12
αδ	39	123	131	293
α	48	19	7	74
δ	10	14	4	28
Nil	24	14	3	41
Total	123	172	153	448

Correlation to resistance to antibiotics The distribution according to phage type and resistance to antibiotics is shown in Table 3

Within phage groups I and II no correlation between the antibiogram of the strain and its protein A production could be demonstrated The distribution within phage group III is shown in Table 4 There was a

TABLE 3 *Distribution of 393 Strains of* S aureus *According to Phage Type and Resistance to Antibiotics*

Resistance in antibiotics	Phage type Group I	Group II	Group III	Total
Sensitive	23	16	15	54
P	14	14	34	62
PS	18	0	3	21
PST	2	0	42	44
PSTM	0	0	50	50
PSTE	0	0	46	46
PSTEM	0	0	46	46
PSTEN	0	0	70	70
Total	57	30	306	393

Capitals indicate resistance to penicillin (P), streptomycin (S) tetracyclines (T), erythromycin (E), methicillin (M) and neomycin (kanamycin) (N)

TABLE 4 *Distribution of 303 Strains of* S aureus *from Phage Group III According to Protein A Reactivity in the Fluorescent Antibody Test and Resistance to Antibiotics*

Resistance to antibiotics	Fluorescent antibody test Positive	Variable	Negative	Total
Sensitive	5	7	3	15
P	14	13	7	34
PST	16	10	16	42
PSTM	2	22	26	50
PSTE	4	10	32	46
PSTEM	2	11	33	46
PSTEN	30	39	1	70
Total	73	112	118	303

Capitals indicate resistance to penicillin (P) streptomycin (S) tetracyclines (T) erythromycin (E) methicillin (M) and neomycin (kanamycin) (N)

significant accumulation of pA pos strains within the group of strains marked PSTEN and the majority of strains exhibiting the antibiograms PSTM PSTE and PSTEM were pA neg

DISCUSSION

The prevalence of pA pos strains of *S aureus* was found to vary in time In 1961/62 10 per cent of the strains produced protein A in an amount equal to that produced by *S aureus* Cowan type 1 (NCTC 8530), while about 25 per cent of the strains produced little or no protein A Four years later the percentage of weak producers among strains received at the Department of Hospital Infections had increased considerably This change coincided with a shift in dominant phage types in Denmark, this has been described by *Jessen et al* (11) and *Bulow* (3) During the past decade the epidemic type complex 52 52A 80 81 belonging to group I had gradually been replaced by group III The intruding strains of group III were susceptible to the phages 83A 84 85 6537 87 and had the antibiogram PST The descendants of strains susceptible to this complex showed a concomitant loss of susceptibility to some phages within the complex and changes in the antibiogram The first population which was small and transient had the antibiogram PSTEN and it was replaced by strains with antibiograms PSTM and PSTEM (rarely PSTE) (3) PSTEN strains are all protein A producers and the majority of PSTM and PSTEM strains are pA neg (see Table 4) *Bulow* obtained experimental evidence (3) which suggested that the blocking of susceptibility to some of the phages within the complex and the change in antibiogram is caused by lysogenization The simultaneous change in protein A production has as yet not proved to be a phage dependent event

Thus the difference in prevalence of pA pos strains in various studies (8 6 12 13) can be explained partly by differences in sensitivity of the methods used for demonstration of protein A and partly by the influence of the origin of the material investigated i e the dominant phage complex in a particular area at a given time The lack of any demonstrable correlations to protein A production in the material studied by Kron

vall et al (12) is probably due to the same factors In addition, the number of strains which they studied in detail was comparatively small

The ability to form large amounts of protein A was shown to be correlated to a deficient haemolysin production Of 123 pA pos strains only 39 showed the predominant αδ haemolysin pattern Almost all of these 39 strains belonged to phage group III and had the antibiogram PSTEN, in 1967 such strains had practically disappeared from Denmark (3) The proportion of pA pos strains producing δ haemolysins is therefore at present in the region of 15 per cent (13/84, see Table 2), while 93 per cent of pA neg strains produce this haemolysin In other words growth of staphylococci on ordinary 5 per cent horse blood agar plates without haemolysis indicates that the strain may be pA pos

When strains were selected for the present material a good representation of protein A producers was required From the figures mentioned above it is evident that the selection led to a high proportion of haemolysin defective strains compared to those employed in other materials (4 10)

A possible pathogenic influence of protein A might be revealed if the outcome of infections caused by pA pos strains could be compared with that of infections caused by pA neg strains provided that the strains were identical in other respects In the present material there are only two subgroups within phage group III which allow of a rough estimate namely those with the antibiograms PSTEN and PSTEM (see Table 4) The protein A production is significantly different in the two groups but they are alike with respect to haemolysin pattern (αδ) Tween 80 reaction and resistance to mercury The difference in resistance to antibiotics is accompanied by a difference in susceptibility to phages within the complex 83A 84 85 6557, 89 These strains belong to the material used in the study of staphylococcal bacteriaemia published by *Jessen et al* (11) the mortality rate for patients infected with these two groups of strains was the same and the two groups of patients were comparable with respect to age and origin of infection Thus, analysis of the present material revealed no demonstrable or suspected pathogenic properties of protein A

My warm thanks are due to *Kirsten Rosendal* Head of the Department of Hospital Infections for supplying the strains together with information about phage type, Tween-80 reaction and resistance to mercury and also for her helpful discussions throughout the study I am indebted to Mrs *Lene Berthelsen* for skilful technical assistance

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THE IMMUNE RESPONSE IN A PATIENT TO AN INFECTION WITH *BACTEROIDES FRAGILIS* SS. FRAGILIS AND *CLOSTRIDIUM DIFFICILE*

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The immune response in a patient to an infection (a perirectal abscess) with *Bacteroides fragilis* ss fragilis and *Clostridium difficile* was investigated with the use of agglutination tests, the Ouchterlony technique and indirect immunofluorescence (IFL) technique Antibodies against *B fragilis* ss fragilis were demonstrated with all the three methods while antibodies against *C difficile* were recorded only with indirect IFL technique A significant decrease of the antibody titres (fourfold or more) as measured with agglutination tests and indirect IFL tests and a disappearance of precipitating antibodies as demonstrated with the Ouchterlony technique were observed during a five month period Serum specimens from blood donors serving as controls showed no reactivity with the antigens used The results also showed antigenic differences between strains of *B fragilis* ss fragilis indicating the possibility of more than one serotype within this subspecies of *B fragilis*

Recently improved techniques for the isolation and cultivation of anaerobic bacteria have shown that the presence of these bacteria in various infections in man is more common than is usually realized (9) Three recent papers have indicated that the *Bacteroidaceae* account for a considerable fraction of the anaerobes isolated from patients with bacteraemia (3, 5 6)

Our knowledge about the host parasite interactions involved in infections with anaerobic bacteria is rather limited In the available literature, we have not been able to find information regarding the immune response in infections in man with the *Bacteroidaceae* or other anaerobes In the present report we will present observations on the immune response in a patient to an infection with *Bacteroides fragilis* ss. fragilis and *Clostridium difficile* the immune response being measured with agglutination tests the Ouchterlony technique and the immunofluorescence technique

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MATERIALS AND METHODS

Case Report

A fifteen year-old girl (B L) with Crohn s disease of the chronic type in the terminal ileum and left colon (confirmed by X ray) was hospitalized

October 19, 1970 at the Department of Surgery, Central County Hospital in Örebro, Sweden, because of diarrhoea and a perirectal abscess that was complicated with fever and penetration of the abscess into the left buttock A surgical incision was made on October 23 and a considerable amount of purulent matter was obtained Within a couple of days the temperature dropped to normal, the diarrhoea stopped and the patient felt well

Bacteriological Studies

Material for bacteriological examination was collected from the perirectal abscess A direct smear of the pus showed Gram negative rods, Gram positive rods, and small Gram positive cocci The pus was inoculated immediately after collection onto blood agar, chocolate agar, and Eosin Methylene Blue (Difco) agar plates for aerobic culture, and onto freshly prepared anaerobic culture media that was less than two hours old The anaerobic isolation media included blood agar prepared from brain heart infusion agar (Difco) with 0 5 per cent yeast extract and 5 per cent sheep blood, Fugonagar (4), and laked blood agar prepared from brain heart infusion agar (Difco), 0 5 per cent extract, haemin, Vitamin K, 5 per cent laked sheep blood, and 4 per cent agar The anaerobe plated media were incubated immediately under strict anaerobic conditions and the plates were examined for growth at two and seven days At two days, abundant growth of anaerobic Gram negative rods, identified as *B fragilis* ss fragilis, and anaerobic Gram positive rods, identified as *C difficile*, was obtained Growth of *Staphylococcus epidermidis*, *Escherichia coli*, and *Propionibacterium acnes* was also obtained, although these latter three organisms were not included in the serologic study The infection was regarded as a mixed aerobic and anaerobic infection

Identification of the anaerobes by the methods outlined by *Cato et al* (7), was performed in the Department of Pathology, Emory University Atlanta, Georgia, U S A Fermentation tests were performed in pre reduced, anaerobically sterilized media from Robbin Laboratories, Chapel Hill North Carolina U S A Gas chromatography was performed on the Beckman GC 2A gas chromatograph from an ether extract of a 48 hour glucose broth culture A complete record of the results of fermentation tests and gas chromatography can be obtained on request from the authors

Serological Procedures

A serum specimen (BL I) was obtained from the patient 14 days after her admission to the hospital Additional serum specimens (BL II, III and IV) were obtained 2 weeks, 10 weeks, and 5 months later Serum specimens from 10 blood donors, selected at random, were included as controls Preparation of antigens and performance of serologic tests were carried out as follows

a) *Preparation of antigens* The isolated strains of *B fragilis* ss fragilis and *C difficile* were cultured for 48–72 hours and then harvested in saline A stock laboratory strain of *B fragilis* ss fragilis was handled in the same way Whole bacterial cells, treated with 3 per cent formalin for 1 hour or boiled for 1 hour, were used as antigens in the agglutination tests at a concentration of approximately 10^9 organisms per ml Whole bacterial cells untreated and suspended in saline were used as antigens in immunofluorescence (IFL) tests in a concentration of approximately 1×10, about 0 005 ml of a bacterial suspension was smeared on a glass slide within a circular area 10–15 mm in diameter Bacterial cells—used as antigens in agar gel diffusion (AGD) tests—were disrupted with an MSE 100 Watt ultrasonic apparatus operated at maximum efficiency The bacteria were suspended in saline at a concentration of 100 mg/ml (wet weight) and subjected to intermittent ultrasonic treatment for a 10 minute period After this treatment, more than 90 per cent of the cells were lysed

b) *Serologic assays* Agglutination tests indirect IFL tests, and AGD tests were carried out as follows

(i) *Tube agglutination* Tests were performed with serum specimens diluted twofold in saline starting at a dilution of 1 10 Five tenths ml of the appropriate antigen was added to 0.5 ml of diluted serum The tubes were incubated at 45° C and the reactions read after 18 hours The highest serum dilution giving visible agglutinations was considered as the agglutination titre

(ii) *Indirect IFL* Tests were performed in the following way 0 03 ml of twofold dilutions of the serum specimens starting at a dilution of 1 10 were used to cover smears of the appropriate bacteria The slides were incubated in a moist chamber at room temperature for 30 minutes, then washed in phosphate buffered saline for 10 minutes The smears were covered with 0 03 ml of a 1 30 dilution of sheep anti human IgG labelled with fluorescein isothiocyanate (FITC) (The conjugate obtained from the National Bacteriological Laboratory, Stockholm, had a molar F/P ratio of 2 0 and an antibody content of 0 8 mg/ml) The slides were incubated for 30 minutes washed in PBS and mounted under a cover glass with glycerine buffered with PBS as mounting fluid The slides were read in a Zeiss fluorescence microscope equipped with a darkfield condenser and an Osram HBO 200 as the lamp source BG 3 was used as the primary filter in combination with Zeiss 47 and 65 as secondary filters A 3+ to 4+ reaction (2) was considered as positive

TABLE 1 *Results of Agglutination Tests, Indirect Immunofluorescence Tests, and Agar Gel Diffusion Tests between Antigens from Three Anaerobic Bacterial Strains and Serum Specimens from Patient BI and Ten Blood Donors*

Serum specimen	Agglutination titres with			Titres in indirect IFL tests with			AGD reactions with		
	Bacteroides fragilis ss *fragilis**	*Clostridium difficile*	*Bacteroides fragilis* ss *fragilis*§	*B fragilis* ss *fragilis**	*C difficile*	*B fragilis* ss *fragilis*§	*B fragilis* ss *fragilis**	*C difficile*	*B fragilis* ss *fragilis*§
BL I	1 80	<1 10	<1 10	1 320	1 [illegible]	≤1 10	+	—	—
BL II	1 80	<1 10	<1 10	1 320	1 160	≤1 10	+	—	—
BL III	1 80	<1 10	<1 10	1 320	1 80	≤1 10	(+)	—	—
BL IV	1 20	<1 10	<1 10	1 80	1 20	≤1 10	—	—	—
Ten blood donors	<1 10	<1 10	<1 10	≤1 10	≤1 10	≤1 10	—	—	—

* Patient s isolate

§ Stock laboratory strain

(11) *Agar Gel Diffusion* AGD tests were performed with Wadsworth s micromodification of the Ouchterlony technique (8) Ultrasonic treated antigen was used at a concentration of 15 *mg/ml* as compared with the wet weight of whole bacterial cells Appropriate wells of the AGD plates were filled with antigen and undiluted serum The plates were left at room temperature and read after 48 hours They were then washed dried stained with Amido black in the usual way and recorded

RESULTS

The results obtained with agglutination tests indirect IFL tests and AGD tests are summarized in Table 1

It will be seen that serum specimen BL I gave an agglutination titre of 1 80 with *B fragilis* ss fragilis The same titres were obtained with specimens taken 2 and 10 weeks later while 5 months later the titre had dropped to 1 20 There was no difference of the titres with formalin treated or boiled antigen No agglutination reactions were obtained with *C difficile* or the laboratory stock strain of *B fragilis* ss fragilis as antigens The blood donors sera gave no agglutination reactions with any of the three bacterial strains tested

In indirect IFL tests, serum specimens BL I, II, and III gave a titre of 1 320 with *B fragilis* ss fragilis and a titre of 1 80–1 160 with *C difficile* Five months after the first serum specimen was taken, these titres had dropped to 1 80 and 1 20, respectively No positive reactions were observed with the stock strain *B fragilis* ss fragilis The blood donors' sera gave no positive reactions in IFL tests with any of the strains

In AGD tests serum specimens BL I and II gave a distinct precipitin line with a reaction of identity with the ultrasonic treated antigen of *B fragilis* ss fragilis but not with antigens of *C difficile* or the stock strain of *B fragilis* ss fragilis Serum specimen BL III gave a faint precipitin line while serum BL IV gave no reaction All blood donors sera were negative

DISCUSSION

Experience with improved culture techniques for anaerobic bacteria have shown that they may occur frequently in fistulas and wound abscesses associated with gastro intestinal disorders, *i e*, in Chron s disease, or in post operative complications following surgery of the gastro intestinal tract or other organs in the abdomen The involvement of certain

anaerobes in certain types of infectious processes, including abscesses, has also been reported by *Finegold* (5) and *Bornstein* (1). While isolates of anaerobic bacteria from blood, recently reviewed by *Finegold* (5), *Gelb* & *Seligman* (6), and *Felner* & *Dowell* (3), have pathogenic significance, it has been more difficult to assess the importance of specific anaerobic isolates from other sources, i.e. fistulas and wound abscesses since, in many instances, multiple anaerobes occur in one infection with or without aerobic bacteria. The findings in the present report showed, however, the occurrence of an immune response to anaerobic bacteria isolated from a perirectal abscess, which gives support for the pathogenic significance of the anaerobes in infections other than bacteraemia or septicaemia.

It is of interest to note that antibodies against *B. fragilis* ss. fragilis were demonstrated both with agglutination tests, indirect IFL tests, and the AGD tests, while antibodies against *C. difficile* were recorded only with the indirect IFL test. Antigens of the *Bacteroides* strain responsible for the agglutination reaction seem to be heat resistant. The results of the present report also show antigenic differences between the two strains of *B. fragilis* ss. fragilis, indicating the possibility of more than one serotype within this subspecies of *B. fragilis*. These findings were confirmed with antisera from rabbits immunized with these bacteria. The antigenic pattern of certain anaerobic bacteria isolated from the gastro-intestinal tract and various infections of man is now in progress for further investigation.

Appreciation is expressed to Mrs. *Judy Gross* and Mrs. *Madeline Freeman* for their laboratory assistance in the identification of the anaerobes.

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SUBACUTE SCLEROSING PANENCEPHALITIS: ISOLATION AND ULTRASTRUCTURAL CHARACTERIZATION OF A MEASLES-LIKE VIRUS FROM BRAIN OBTAINED AT AUTOPSY

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Brain tissue was obtained at autopsy from a patient with subacute sclerosing panencephalitis (SSPE) and kept frozen for nine months prior to attempts at virus isolation. Mixed cultures of trypsinized brain material and Vero monkey kidney cells developed cytopathic changes resembling those associated with measles virus. The cytopathogenic effect (CPE) was transferred by cells, and in later passages, also by culture supernatants. Cells and supernatants from cultures showing CPE agglutinated monkey red cells. Haemagglutination inhibition, immunofluorescent and neutralization experiments showed the cytopathogenic agent to be serologically similar to measles virus. Gel precipitation experiments, on the other hand, indicated differences in antigenic composition between the SSPE cultures and cultures of a vaccine (Edmonston) strain of measles virus. Ultrastructural studies revealed cytoplasmic inclusions of nucleocapsids in the SSPE cultures. The appearance and diameter of the SSPE nucleocapsids differed from those of the vaccine strain. Budding and complete viral particles were seen in both SSPE and vaccine strain cultures. It is concluded that the SSPE agent isolated here is most probably a measles virus but in some respects different from the Edmonston strain of measles virus.

Subacute sclerosing panencephalitis (SSPE) is to-day a preferred term for the condition described by *Dawson* (10, 11) as 'inclusion body encephalitis', by *van Bogaert* (6) as "subacute sclerosing leukoencephalitis", and by *Pette & Döring* (24) as „nodular panencephalitis".

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SSPE has recently attracted much interest because of its possible relationship to infection with measles virus, presumably acquired a long time prior to the onset of symptoms. Although a viral etiology was suggested already by *Dawson* in 1933 (10), the first evidence was afforded in 1965 by *Bouteille et al.* (7), by their ultrastructural demonstration of myxovirus like particles in SSPE brain tissue. In 1967 *Connolly et al.* (9) reported elevated titres of measles antibody in serum

and cerebrospinal *fluid* (*CSF*) and the presence in brain tissue of measles antigen

Recently, several groups of investigators have reported successfull propagation of a measles-like virus from SSPE brain specimens (3, 8, 14, 16, 22, 23) Common to all these studies has been the establishment of primary brain cell culture, which has been assumed to be a prerequisite for the propagation of the agent (23) It has further been shown that the SSPE *cytopathogenic* agent could be *isolated* by cocultivation of brain cells and various continuous line cells (3, 14 16)

In the present communication we report the isolation and ultrastructural characterization of a measles-like virus from brain tissue obtained at autopsy in a case of SSPE

MATERIAL AND METHODS

Case Report

A 14 year old girl (R R 1408/69) was admitted to the Department of Neurology Rikshospitalet, Oslo, in June 1969 after 6 months of gradual intellectual deterioration increasing speech and gait difficulties and attacks of petit mal and *myoclonus Seven months prior to the onset of* symptoms, she had suffered bilateral optic neuritis, which after recovery left the vision on the left eye impaired

Neurological examination revealed signs of gross cerebral involvement, the most significant signs being a pronounced amnestic dementia dysphasia and dyspraxia as well as bilateral spastic paresis and ataxia Rightsided myoclonus and infrequent petit mal like attacks were observed Ophtalmoscopy showed bilateral peripapillar hyperpigmentation and pallor of the left optic disc

Electroencephalography (EEG) was abnormal with periodic complexes of slow waves of high amplitude in all head regions The CSF was acellular with a total protein content of 75 mg per cent Gammaglobulins were elevated (33 per cent of total protein) and exhibited several distinct fractions of restricted heterogeneity on electrophoresis Measles antibody titres in serum and CSF determined by haemagglutination inhibition (HI) were elevated and rose to terminal titres of 1 8192 and 1 128 respectively

Therapy with Prednisone and then with Prednisone and *Azathioprine* (*Imurel*) *for three weeks* seemed to slow down the progression of the disease and was accompanied by a transient fall in serum measles antibody titre However a decorticate coma gradually developed, and she died approximately 10 months after the onset of symptoms The patient had had measles at the age of 4 She had not received measles vaccine

Postmortem Examination

Autopsy was performed three hours after death Apart from *extreme emaciation* and bronchopneumonia, the significant findings were *confined to* the central nervous system The meninges were slightly thickened The brain weighed 1200 g and showed narrowed gyri and widened sulci The cortex was generally atrophic and the gray white junction obscured

Microscopical examination showed loss of neurons and extensive demyelination Marked gliosis was *found* in all regions examined with increase of both astroglia and *microglia* Both hemispheres and parts of the brain stem showed diffuse and perivascular infiltration of mononuclear cells mainly lymphocytes and plasma cells Eosinophilic cytoplasmic inclusion bodies were seen in a number of *neurons and a few glial cells but no intranuclear* inclusions were observed (Neuropathological examination performed by dr *Augot Christie Løken* Section of neuropathology Rikshospitalet Oslo)

Brain Material

Brain tissue was obtained three hours post mortem Samples were immediately frozen and stored at —70° C without any preservative treatment After 9 months of storage representative specimens from the left parieto-occipital region were thawed minced and trypsinized according to standard procedures Dispersed cells were washed three times with Hanks balanced salt solution (BSS) and resus*pended in medium* 199 supplemented with 20 per cent inactivated calf serum $NaHCO_3$ and antibiotics

Cultivations

Brain cell suspensions in *15 ml aliquots* containing 5×10 cells *per ml* were dispersed in tissue culture bottles which were sealed and incubated stationary

Vero (African Green Monkey kidney continuous line) cells were grown in medium 199 with 5 per cent inactivated calf serum and maintained with 2 per cent calf serum Passages of Vero cells were made according to standard procedures

Approximately 5×10^5 brain cells and 2×10^n Vero cells were grown in medium 199 *with 10 per* cent calf serum 0 1 per cent $NaHCO_3$ and antibiotics Brain cells were obtained either from *fresh* trypsinized samples or from five day old brain cell cultures These samples included non attached cells and the medium Mixed cultures were *maintained* in 199 medium with 2 *per cent* serum The medium was renewed every two or three days Sub-

cultures were made every 10 days by brief *trypsin* treatment of the monolayers Equal numbers of fresh Vero cells were added to each passage Fresh Vero cells were also inoculated with various volumes of supernatant from subcultures at each passage level Non inoculated controls were included in all passages

All cultures were regularly examined for cytopathogenic effect (CPE) Samples from the various passage levels were transferred to Leighton tubes and cultivated as described above Flying coverslips which were placed into the tubes were removed after various times of incubation The coverslips were fixed in acetone and stained with haematoxylin-eosin (HE)

Virus

Edmonston A strain of measles virus obtained from Dr *E Norrby*, Karolinska Institutet, Stockholm was propagated in stationary cultures of Vero cells Supernatants were removed when CPE was seen in approximately 2/3 of the cells, clarified by centrifugation at 800 × G for 15 minutes and stored at —20° C Infectivity titres were established in Vero cells by the end point dilution method (see below)

Haemagglutination (HA) and Haemagglutination Inhibition (HI)

Haemagglutinating antigen was prepared by Tween Ether extraction (TE antigen), according to *Norrby* (18), and stored at 4° C In some experiments, clarified tissue culture supernatants, concentrated 20 × by Polyethylene Glycol (mw 4000) followed by dialysis against physiological saline, were used The microtest of *Sever* (26) was used, employing 0 5 per cent cercophitecus erythrocytes Cell pack antigens were prepared by disrupting the cells with osmotic shock, and by freezing and th aw nb twice Four haemagglutinating units of antigen were used in the HI test Serum from a rabbit immunized with Edmonston strain TE antigen was used as standard reference

Infectivity and Neutrali ation Tests

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were inoculated into 5 tubes, 0 2 ml each The tubes were incubated in a roller at 37° C for a period of 10 days Neutralization assays were performed using 100 tissue culture infectious doses ($TCID_{50}$) of virus and rabbit TE *antiserum* In some experiments, serum from the patient was employed All experiments included controls with known Edmonston virus Virus and sera were incubated at room temperature for one hour and at 4° C overnight before being added to *the cell cul*tures

Immunoprecipitation Tests

These were performed in the double diffusion technique in 1 per cent agarose (A-45 Industrie Biologique Française) in Veronal buffer, pH 8 6, ionic strength 0 05, at room temperature Sonicated cell pack antigens were prepared from Edmonston strain cultures and SSPE cultures exhibiting CPE, as described by *Panelius et al* (21) Control antigen from non infected culture cells was prepared in the same way The antigen was allowed to react with sera for 36 to 48 hours Sera used were 1) serum from a child without known exposure to measles infection and in whom HI serology was negative, 2) a pool of normal human sera (HI titre 128) and 3) serum from the patient as well as from three other cases of SSPE The HI titres in these sera varied from 2048 to 8192

Fluorescent Antibody Tests

Monolayer cultures were trypsinized and the cells washed in PBS Diluted cell suspensions were spun down on microscope slides at 1500 RPM for 10 minutes in a Shandon Cytocentrifuge, giving preparations of evenly dispersed cells The slides were air dried and fixed in acetone for 10 minutes at 4° C Fluorescent antibody experiments were also in some cases performed directly on monolayer cultures on Leighton tube coverslips

Indirect immunofluorescent experiments employed dilutions of the sera described in the preceding section, and fluorescein · isothyocyanate (FITC) conjugated rabbit antiserum against human immunoglobulin G (IgG) The FITC conjugation was performed according to *Nairn* (17) Direct immunofluorescent experiments were done with FITC conjugated IgG from the patient s serum at an IgG concentration of 0 5 mg per ml

Appropriate blocking procedures were included in all experiments Microscopy was done on a Leitz research microscope (ORTHOPLAN), with ultra high pressure mercury lamp and vertical illuminator Photographs were taken in an ORTHOMAT microscope camera using Ansco 500 daylight film

Electron Microscopy

Washed cell packs were fixed in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer pH 7 2 for 1 2 hours and postfixed in 1 per cent osmium tetroxide in Tyrode buffer, pH 7 2, for 1 hour Dehydration was carried out in graded ethanol, and the specimens were embedded in Epon 812 Thin sections were cut with an LKB ultramicrotome, and stained with lead citrate and uranyl acetate The sections were examined in a Siemens Elmiskop I electron microscope

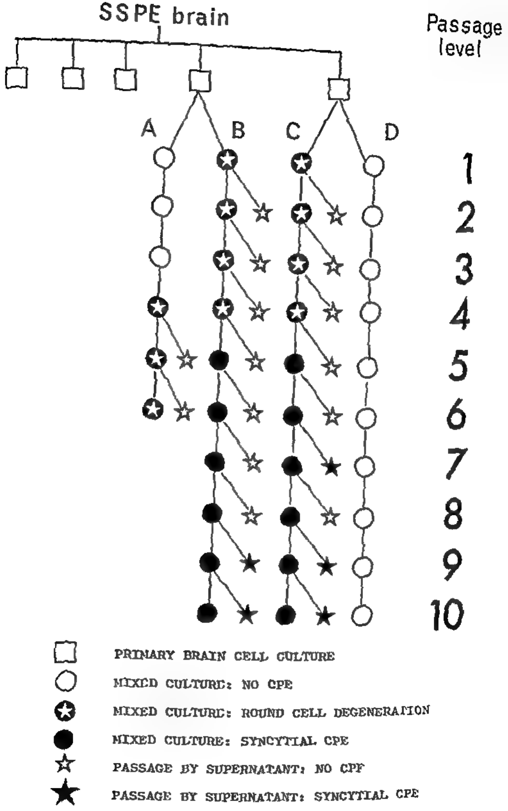

Fig 1 Passages of mixed cultures of brain cells and Vero monkey kidney cells See text

RESULTS

Brain cell cultures Only a small fraction of the cells derived from the brain samples attached to the surface of the culture vessels No multiplication of the cells could be observed during the 28 days cultivation period, nor did CPE appear Attempts to subcultivate these cells were not successful

Mixed culture Four culture lines were initiated (Fig 1) CPE, characterized by localized round cell degeneration, was observed in some first passage cultures, and could be transferred to further passages by the cells CPE negative first level cultures showed round cell degeneration in later passages (Fig 1) Small syncytia appeared from the 5th subculture in two culture lines The syncytia were few and small when first observed, but were more numerous and developed into syncytial giant cells in later passages The extent of syncytial development, however, never reached the same degree as that seen in Edmonston virus cultures

Transfer of CPE by culture supernatant was first observed at the 6th passage level in one line, and at the 8th level in another line (Fig 1) In subsequent passages CPE was regularly transmitted by the supernatant of these cell lines Although equal infective doses were used, the CPE in these cultures developed more slowly than in the vaccine strain cultures Syncytia usually appeared 4–5 days after inoculation and reached maximum development after 9–12 days, a 2–3 day delay compared to the vaccine cultures Syncytia and syncytial giant cells showed diffuse increase in eosinophilia of the cytoplasm, and in some cells eosinophilic intracytoplasmic inclusion bodies were seen No intranuclear inclusions were observed

Biological activities HA-positive cell homogenates were seen on one cell line (line B, Fig 1) from the first passage level, and in a second line (line C) from the fifth level The others remained negative Unconcentrated supernatants were HA negative until the 11th passage of line C, which gave a weakly positive reaction In concentrated supernatants, however, HA was observed from the 7th passage in line B HA specificity was ascertained in the HI test, where rabbit TE-antiserum completely inhibited the HA activity, wereas control rabbit serum did not No difference in HI titre was found for the rabbit TE antiserum, nor for the patient's serum, when these sera were tested against the Edmonston strain virus and the SSPE agent

TABLE 1 *Haemagglutination Inhibition (HI) and Neutralization (NT) Titres of Sera from the SSPE Patient and from a Rabbit Immunized with Edmonston Strain Measles Virus against the SSPE Agent and the Edmonston Virus*

Virus	Antiserum against Edmonston virus HI	NT	SSPE agent HI	NT
Edmonston	256	640	256	320
SSPE	256	20	256	640

Table 1 shows that both rabbit TE-antiserum and serum from the patient neutralized the infectivity of Edmonston strain and of SSPE cultures While serum from the patient showed no significant difference in neutralizing titre, the titre of rabbit TE-antiserum was considerably lower for the SSPE-agent than for the Edmonston strain

In the fluorescent antibody method, strong apple green fluorescence was seen in approximately 60–90 per cent of the cells in the Edmonston strain cultures In some of the cells, small aggregates of staining material were seen within the nucleus In most cells, however, the staining was cytoplasmic, often with speckled or lumpy appearance In the SSPE cultures, the degree of sta
always considerably weaker In e
sages, approximately 20 per cent of
stained In later passages, staining
in up to 60 per cent of cells The
was cytoplasmic, partly of a diffuse,
a speckled nature (Fig 2). No
clear staining was seen Blocking
abolished the fluorescence, both in
and the indirect systems None

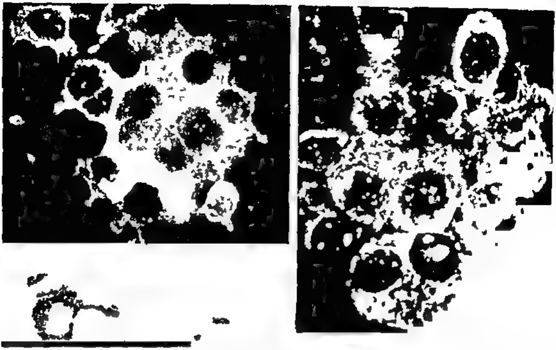

Fig 2 Cytocentrifuge preparations of cells from SSPE culture line II 11th passage level (left) and from Edmonston strain culture (right) showing syncytial giant cells stained in the indirect immunofluorescent method with serum from patient R R Note the weaker staining of the SSPE culture and restriction of staining to the cytoplasm while Edmonston strain infected cells contain inclusions of staining material also within the nucleus (× 1200)

employed stained non infected culture cells No significant difference in staining pattern between the SSPE sera used was seen The normal human serum pool gave a moderate cytoplasmic fluorescence when used undiluted, but did not reveal the intranuclear staining seen with SSPE sera in some of the Edmonston strain culture cells The measles negative control serum did not stain any of the cultures

In the immunoprecipitation experiments 3–5 precipitation lines developed when the various SSPE sera reacted with sonicated cell pack antigen from Edmonston strain cultures The main line was visible in 1 128 the others in 1 16 dilution Pooled normal human serum gave only one very weak precipitation line, visible in 1 2 but not in 1 4 dilution None of the sera reacted with control antigen Further details concerning immunoprecipitation in SSPE and other diseases will be published elsewhere

Cell pack antigen from SSPE cultures failed to react with any of the sera until the 10th passage Antigen prepared from this and later passages gave one hazy precipitation line with one of the four SSPE sera (Fig 3) Unexpectedly, this serum was not from the patient from whom the SSPE agent was isolated, but from a 14 year old male with an unusually protracted course of illness The hazy line developed by this serum did not appear to fuse with any of the lines developed against the vaccine strain antigen

Electronmicroscopic investigations showed that control cultures were usually well preserved as judged from the appearance of mitochondria and the rough endoplasmic reticulum (Fig 4) The nuclei and cytoplasm contained no structures resembling nucleocapsids Microvilli were often observed at the surface of the cells The plasma membrane had a normal three laminar appearance with a diameter of 80 to 100 Å

Cells infected with the Edmonston strain virus contained large aggregates of *nucleo-*

capsid like structures in the cytoplasm (Fig 5) The aggregates were located to the cytoplasmic matrix and were often associated with lipid droplets They varied in size from those containing a few osmophilic strands to those filling a great portion of the cytoplasm The diameter of the single strands, which appeared curved or twisted varied from about 450 Å to 550 Å They had a characteristic "fuzzy" appearance (Fig 6), which made it difficult to determine the exact thickness and to define any substructures At low magnifications the cell plasma membrane appeared thickened in some areas This was particularly evident at sites where the plasma membrane was curved outwardly (Fig 5) At higher magnification these thickenings could be identified as viral material

The budding phenomenon was regularly seen Free particles were found near the cell surface (Fig 7) They varied in size from about 3000 Å to about 7000 Å The surface appeared to contain a "fuzzy" coat with projections arranged vertically to the membrane (Fig 7) Particles with a diameter of 450 to 550 Å could be seen immediately underneath the membrane in some areas while in others only a fuzzy coat could be seen (Fig 7) Hollow tubules with an inner diameter of about 300 Å associated with the coat lining the inside of the membrane could also be seen No intranuclear inclusions of nucleocapsid like material were observed

Cells infected with the SSPE agent showed various degrees of damage from vacuolization to almost complete destruction Some of the cells contained cytoplasmic inclusions of strands resembling nucleocapsids (Fig 8) The strands were usually found in aggregates located either to the cytoplasmic matrix or in cytoplasmic vacuoles (Fig 8) The appearance of these strands differed from those observed in Edmonston strain virus cultures The outer diameter was about 130 Å and the surface lacked the "fuzzy" coat found for Edmonstone strain nucleocapsids (Fig 9) Both in cross and longitudinal sections some strands appeared as hollow tubules with an inner diameter of about 40 Å while other

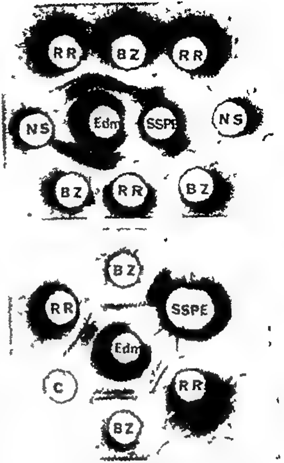

Fig 3 Immunoprecipitation experiment Sonicated cell pack antigens from SSPE (SSPE) and Edmonston (Edm) strain cultures in center wells C Control antigen NS Normal human serum (pool) Above The patient's serum (R.R) and serum from another SSPE case (B Z) give four to five visible precipitation lines against the vaccine antigen No visible reaction is seen between serum R R and the SSPE antigen while serum B Z gives a hazy precipitation line Below Increasing the amount of SSPE antigen five times increases the precipitation line with serum B Z but no visible line is seen against serum R R

strands appeared compact (Fig 9) In longitudinal sections these structures tended to show a periodic cross striation

Various stages of budding and free virions were frequently observed (Fig 10) Their diameter varied from about 3000 to 8000 Å Like Edmonston strain virions, the SSPE virions had plasma membranes covered by a coat with short projections or spikes Under-

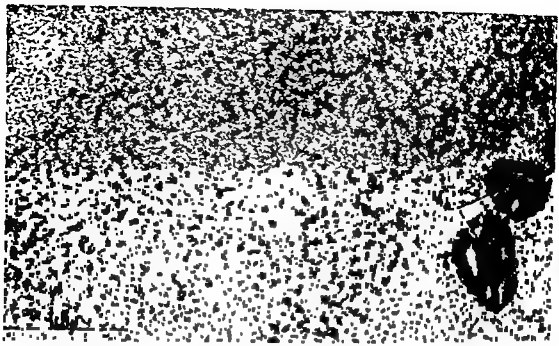

Fig 6 Nucleocapsids in the cytoplasm of Edmonston strain virus infected cell Note the hairy appearance of the nucleocapsids which have a diameter of about 450 Å to 550 Å

the Edmonston strain virus contamination from the laboratory strain does not seem likely

The question whether the agent isolated from SSPE brains is a measles virus or a closely related virus, remains to be finally answered Data from different laboratories seem to agree on some points, but not on others The cytopathology caused by the SSPE agent isolated here was in principle similar to that caused by measles virus However, differences in rate of development and extent of CPE between the SSPE and the Edmonston strain isolates were consistently noted throughout the experiments This is in agreement with the work of *Baublis* & *Payne* (5), who found that their SSPE isolate gave less pronounced CPE than the standard Edmonston strain

The similarity of the SSPE agent to measles virus in antigenic characteristics revealed by HA, HI and immunofluorescent tests, reported by others (5, 8, 15, 16, 23), is evident also in the present study On the other hand, the neutralization experiments would indicate some difference between the two, as rabbit antibody against the haemagglutinin of Edmonston strain virus was less effective in neutralizing the SSPE isolate than the vaccine isolate *Horta-Barbosa et al* (15) found consistently lower neutralizing titres in SSPE sera against SSPE isolates than against wild strain isolates No corresponding difference was found here, when the patient's serum was tested against the autologous and the Edmonston strain isolates This is in accordance with results from others, where no difference of SSPE sera in plaque neutralization between SSPE and Edmonston strain isolates was seen (23) SSPE sera revealed several precipitation lines when reacted with sonicated cell pack antigens from Edmonston strain infected cultures It is not known however, whether the antigens involved represent proteins of virus particles or virus specific non structural proteins developed in the cell cultures during virus infection As multiple precipitation lines in the present study were revealed by SSPE sera only, it would seem safe to assume that they reflect antigenic determinants that have been immunogenic to the patients during the brain infection

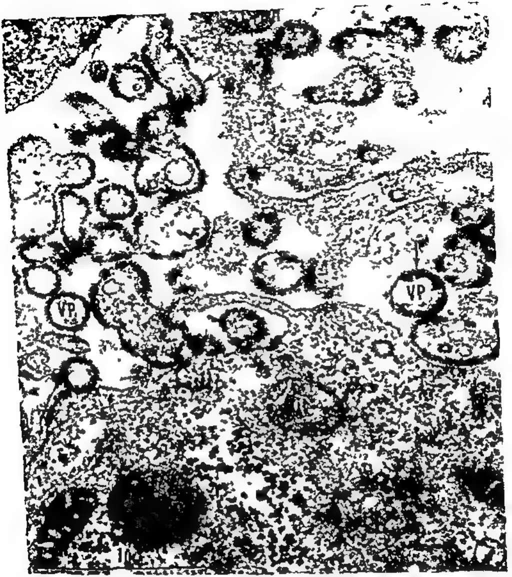

Fig 7 Budd ng phenomenon and virus part cles (VP) at the surface of an Edmonston stra n virus nfected cell. Note the coat (projections) at the surface of the particles (arrows) Granules with a diameter of about 500 Å are present underneath the membrane of the particle (VP at the center right of the p cture

with the SSPE agent The fact that these lines did not appear when the SSPE antigen was used may be due to a too low concen tration of relevant antigenic components in these cultures Some support for this view may be derived from the consistently weaker CPE and immunofluorescent staining seen in the SSPE cultures when compared with the Edmonston strain cultures The hazy pre cipitation line developed by one of the SSPE sera (serum BZ) against SSPE antigen may however not be explained by quantitative factors alone As this line did not appear to fuse with any of the lines revealed against the vaccine strain it would appear that the SSPE cultures contain an antigen not pre sent in the vaccine strain cultures Further one must assume serum BZ to contain an

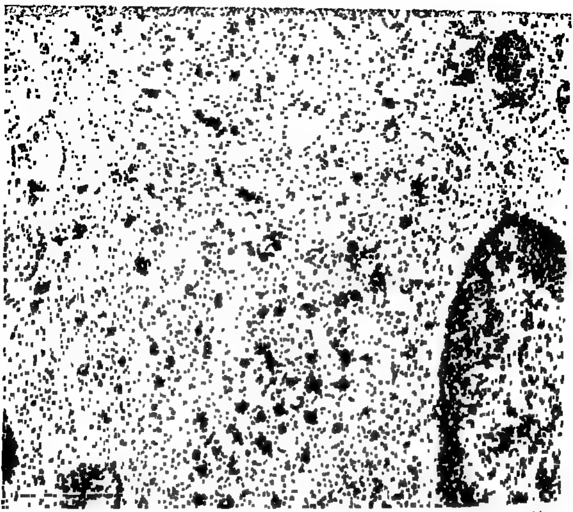

Fig 8 SSPE infected cell containing smooth nucleocapsids in the cytoplasmic matrix Nucleus (N)

antibody not present in the patient's own serum, nor in the other two SSPE sera The difference between serum BZ and the other SSPE sera in this respect may be related to the fact that this patient had an unusually protracted course of illness with slowly progressive mental deterioration over the course of 4–5 years, while the other three SSPE patients all died within 1 year after onset

The ultrastructural appearance of fuzzy strands with diameters from 450 to 550 Å in aggregates in the Edmonston strain cultures correspond to the observations of *Baringer & Griffith* (4), and are assumed to represent nucleocapsids The present study, revealing cytoplasmic inclusions only, is in agreement with their study, whereas *Oyanagi et al* (19, 20) have found intranuclear inclusions of nucleocapsid material in both wild and Ed monston strains of measles virus These dis crepancies may be related to several factors, such as amount of infective virus, cell substrates and number of cell passages used

The nucleocapsid strands observed in the SSPE cultures were clearly different from those seen in the vaccine strain, in their smaller diameter (130 Å) and smooth sur face They resembled closely the "smooth fi laments" described by *Oyanagi et al* (20) in CV-1 cells infected with SSPE viruses These filaments—or rather hollow tubules—were stated to have a diameter of 150 to 170 Å We did not in the SSPE cultures observe structures similar to the larger "granular fila

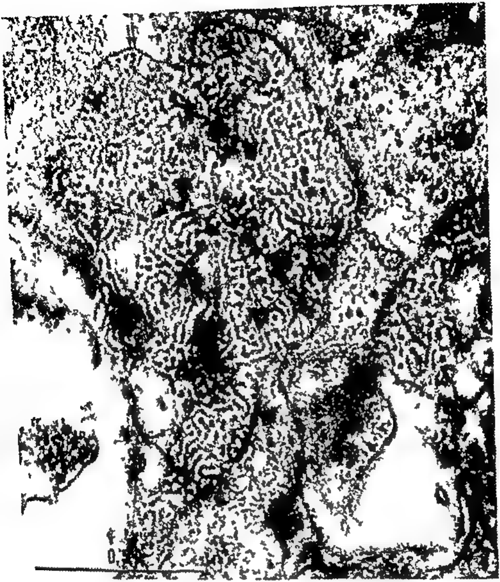

Fig 9 SSPE infected cell with nucleocapsids in a vacuole The limiting membrane of the vacuole is three laminated (indicated by double arrows) The nucleocapsids appear cross striated (single arrow)

ments with diameter from 220 to 250 A described by *Oyanagi et al* (20) for both SSPE viruses and for wild and Edmonston strain viruses Judging from their illustrations however these filaments seem to us larger than stated and could possibly correspond to the strands found by us in the Edmonston strain cultures.

No intranuclear inclusions of nucleocapsid material nor fluorescent nuclear material could be seen in our SSPE cultures These findings are in contrast to those of *Oyanagi et al* (20) and of *Parker et al* (22) who found intranuclear inclusions to be prominent in their SSPE cultures In these reports however the brains serving as source material contained intranuclear inclusions whereas only cytoplasmic inclusions were found in our case Although intranuclear inclusions are a prominent feature of SSPE they are not uni

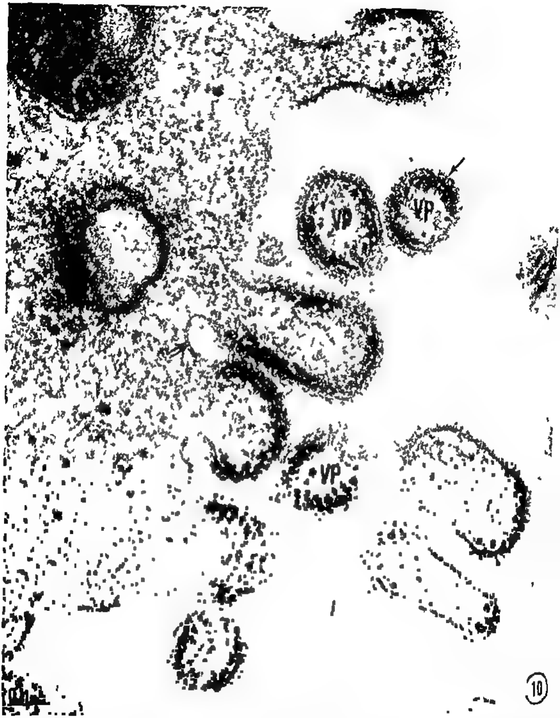

Fig 10 SSPE infected cell with budding and virus particles (VP) at the surface Single arrows indicate projections in the coat of the viral particles Underneath the viral membrane a row of granules is seen, possibly representing uncleocapsid material embedded in a fuzzy coat Double arrows indicate an invagination of the plasma membrane

versally present *Schiött* (25) and *Herndon & Rubinstein* (13) have described cases where nuclear inclusions were present in biopsy material, but could not be demonstrated in autopsies performed only weeks or months later In ultrastructural studies on SSPE brain tissue, the latter authors have suggested that there is a change of location of the viral material from nuclear to predominantly cytoplasmic, as the disease progresses The significance of this sequence of events is unknown The demonstration by *Herndon & Rubinstein* (13) of viral particles budding from the compact cytoplasmic inclusions, suggests that it may reflect maturational stages of the virus If so the stage of illness at which material for virus isolation is obtained, may be very significant for the biological characteristics of the isolate In our case, this may be part of the explanation for both the unusual finding of only cytoplasmic inclusions, and for the finding of apparently complete virus particles in the cultures The latter finding is in some contrast to the findings of *Oyanagi et al* (20), who could not observe complete virions in their SSPE cultures The complete SSPE virions found here did not differ from the Edmonston strain virions except for the smaller size of the nucleocapsids found underneath the particle membrane

In summary the results indicate the principal similarity between the SSPE agent and measles virus The small differences noted in cytopathogenic effect, antigenic composition and ultrastructural appearance of nucleocapsids do not allow a distinction between SSPE and other measles viruses The stage of illness at which material was obtained as well as the frozen state of the source material and the subsequent cultivation procedures are factors that may have influenced the virus characteristics

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THE EFFECT OF MITOMYCIN C ON DNA SYNTHESIS IN P-388 CELLS

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The effect of mitomycin C on the synthesis and degradation of DNA in mouse P-388 cells grown in suspension culture has been investigated. Treatment for 24 hours with 0.5 μg/ml of the antibiotic selectively suppressed DNA synthesis in these cells. DNA synthesis was measured as incorporation of ^{3}H-TdR, and during the first 2.5 hours of treatment the only effect of mitomycin C on this parameter was a reduced incorporation on the DNA polymerization level. However, more than 8 hours after exposure to the antibiotic reduced uptake of this precursor into the cold acid soluble pool was found in the treated cells. Exposure for 1 hour to 0.5 μg/ml of mitomycin C also caused a slightly reduced rate of DNA synthesis initially, but in these cells no significant decrease in total DNA was found 24 hours after the treatment. Mitomycin C induced degradation of DNA was not found during the first 24 hours of treatment.

Mitomycin C is generally accepted as a selective inhibitor of DNA synthesis in microorganisms and eucaryotic cells, and the inhibition is most probably due to alkylation of DNA and a steric hindrance of the replication process (*Szybalski & Iyer 1967*).

Extensive degradation of DNA has been found in bacteria and also in some mammalian cells exposed to the antibiotic (*Shatkin et al. 1962, Kersten & Themann 1962*). However, accumulating evidence indicate that mitomycin C-induced degradation of DNA in bacteria is a secondary phenomenon related to DNA repair processes (e. g. *Boyce & Howard-Flanders 1964, Terawaki & Greenberg 1966*) and recent data (*Rauth et al. 1970*) might suggest that repair mechanisms also exist in mammalian cells.

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In a previous report (*Ørstavik 1972*) data were presented indicating that mouse P-388 cells in tissue culture may recover from growth inhibition and giant cell formation induced by short treatment with mitomycin C, suggesting that repair mechanisms may exist in these cells. As a basis for further studies on DNA repair in mammalian cells exposed to mitomycin C, the effects of the antibiotic on synthesis and degradation of DNA in suspension cultures of P-388 mouse cells have been investigated.

MATERIALS AND METHODS

Cell Cultures

P 388 mouse cells (*Dawe & Potter 1957*) were grown in suspension culture on Eagle's minimum essential medium with 10 per cent foetal bovine serum as described previously (*Ørstavik 1972*).

Labelling with ^{3}H-Thymidine

Pulse labelling of the cells was performed by incubating culture samples with ^{3}H TdR* (*final*

concentrat on 0 1 or 1 0 μCi/ml) at 37 C for 15 minutes the samples were then chilled at 0 C for 5 minutes

For prelabelling the cultures were grown with ^{3}H TdR for 20 hours (5 doses of 0 04 μCi/ml were added at 4 hour intervals)

Cell Fractionation

Culture medium was removed after centrifugation and the cells were washed with cold PBS The cells were then extracted twice with either TCA (5 per cent w/v) or PCA (0 6 N) for 20 minutes at 0 C and subsequently with TCA (5 per cent v/v) for 20 minutes at 90 C Acid insoluble cell material was finally dissolved in 1 N KOH

Thin Layer Chromatography

Cold PCA cell extracts were neutralized immediately with KOH and the perchlorate precipitate was removed by centrifugation The supernatant was chromatographed on 0 2 mm thin layer cellulose plates using isobutyric acid/0 2 N NH_3 (2 1) as solvent and T TdR TMP TDP and TTP as carriers The carrier spots were localized with UV light (Rf values T 0 77 TdR 0 71 TMP 0 49 TDP 0 37 and TTP 0 25) scraped off and eluted with 1 ml of distilled water

Radioactivity Measurements

Radioactivity was measured in a Packard Tri Carb model 3305 liquid scintillation counter From pulse labelled cultures 25 μl of culture medium cell extracts and thin layer chromatogram eluates were counted in 10 ml of toluene containing 4 g/l of PPO 0 05 g/l of POPOP and 15 per cent of etyleneglycol monomethyl ether From prelabelled cultures 1 ml of culture medium cold and hot TCA cell extracts were counted in 10 ml of Insta gel scintillation liquid (Packard Instrument International Zurich

Colorimetric Determination of Nucleic Acids and Protein

The content of nucleic acids was measured in the hot TCA soluble cell fraction DNA was determined as diphenylamine reacting material against deoxyribose Burton 1956) RNA as orcinol reacting material against ribose (Bo n 1946) Protein was measured in the hot TCA insoluble cell fraction with the biuret method (Layne 1957) using bovine albumin as standard.

Chemicals

Mitomycin C was purchased from Kyowa Hakko Kogyo Co Tokyo kept as aqueous stock solution (1 mg/ml) at 4 C for maximum 1 week and added to experimental cultures to the appropriate final concentrations Thymidine 6 T(n) was obtained from The Radiochemical Centre Amersham kept as aqueous stock solution (100 μCi/ml specific activity 3 5 C/m mol) at 4 C and renewed every second month

RESULTS

Effect of Mitomycin C on Production of DNA RNA and Protein

P 388 mouse cells grown continuously with 0 5 μg/ml of mitomycin C and cells exposed to the same concentration for 1 hour followed by growth in pure medium were analysed for DNA RNA and protein 24 hours after the antibiotic had been added It appears from Fig 1 that the amount of DNA in the continuously treated cultures was reduced by about 20 per cent as compared with controls whereas no difference was found between controls and cultures treated with the antibiotic for 1 hour The content of RNA and protein in the cell cultures was not significantly affected by either treatment

Mitomycin C Induced Inhibition of DNA Synthesis

Reduced net production of DNA as measured colorimetrically (Fig 1), might be due to decreased synthesis or increased degradation or both The effect of mitomycin C on the synthesis of DNA was therefore determined by measuring the incorporation of ^{3}H TdR into hot TCA soluble cell extracts

From the results in Fig 2a it clearly appears that DNA synthesis in the P 388 cells was rapidly and strongly suppressed by 0 5 μg/ml of the antibiotic Thus incorporation of ^{3}H TdR was significantly reduced during the first hour of treatment and was inhibited by about 40 per cent after 8 hours of treatment In cells which had been exposed

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* Abbreviations ^{3}H TdR thymidine 6 T(n MC mitomycin C PBS phosphate buffered saline 0 15 M pH 7 2 TCA trichloroacetic acid PCA perchloric acid T thymine TdR thymidine TMP thymidine 5 monophosphate TDP thymidine 5 diphosphate TTP thymidine 5 triphosphate PPO 2 5 diphenyloxazole POPOP 1 4 bis 2(4 methyl 5 phenyloxazolyl) benzene

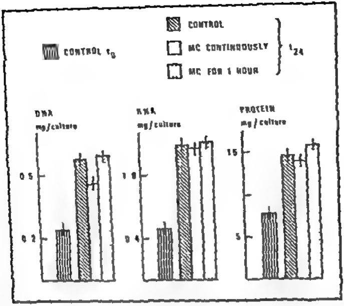

Fig 1 Effect of Mitomycin C on production of DNA, RNA and protein P-388 cells were incubated for 1 hour in pure medium (control), or in medium containing 0 5 µg/ml of mitomycin C, harvested and washed Controls and one half of the treated cells were grown further in pure medium, the rest in medium in which the concentration of antibiotic was maintained The content of DNA, RNA and protein in the cultures was determined as described under materials and methods

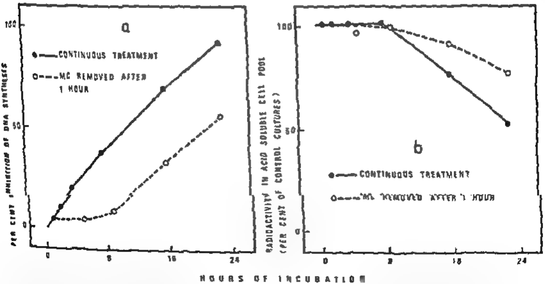

Fig 2 Effect of Mitomycin C on DNA synthesis P 388 cells were grown in medium containing 0 5 µg/ml of mitomycin C continuously or for 1 hour followed by growth in pure medium The cells were pulse labelled with ^{3}H TdR at intervals as indicated a MC induced inhibition of DNA synthesis as measured by radioactivity in hot TCA soluble cell extracts b Uptake of ^{3}H TdR into the cell pool as measured by radioactivity in cold TCA soluble cell extracts For details see materials and methods

to mitomycin C for 1 hour and then transferred to pure medium inhibition of DNA synthesis was only moderate (5 to 10 per cent) during the first 9 hours of incubation

Measurements of the radioactivity in the cold TCA soluble extracts (Fig 2b) revealed that this cell fraction contained the same amounts of radioactivity in control and mitomycin C treated cultures during the first 8–9 hours of incubation This finding shows that the initial suppression of ^{3}H TdR incorporation into DNA was not due to reduced uptake of labelled precursor into the cold acid soluble cell pool

The acid soluble pool of cells exposed to mitomycin C for short periods was further analysed to determine whether the early inhibition of ^{3}H TdR incorporation might be due to an effect of the antibiotic on the phosphorylation of ^{3}H-TdR to the immediate DNA precursor ^{3}H TTP *Table 1 shows that* exposure to 0 5 μg/ml of mitomycin C for 2 5 hours had no effect on the rate of phosphorylation of ^{3}H TdR in the cell pool Less than 1 per cent of the radioactivity in the cold acid extract was recovered as thymine and thymidine, and about 90 per cent was found as TTP in controls as well as in mitomycin C treated cells Similar results were obtained when the concentration of the antibiotic was increased up to 5 μg/ml

TABLE 1 Effect of Mitomycin C on Phosphorylation of ^{3}H TdR

Mitomycin C (μg/ml)	Distribution of radioactivity in cold PCA cell extracts (per cent of total)			
	T + TdR	TMP	TDP	TTP
0 (control)	0 2	3 1	5 9	90 8
0 5	0 6	3 1	4 6	91 7
5 0	0 2	5 0	5 8	89 0

P 388 cells were incubated in pure medium or in medium containing 0 5 or 5 0 μg/ml of mitomycin C for 2 5 hours and subsequently pulse labelled with ^{3}H TdR The thymine thymidine and thymidine phosphates were extracted with cold PCA, separated by thin layer chromatography and the radioactivity measured as described under materials and methods

Delayed Effect of Mitomycin C on Cell Uptake of ^{3}H-TdR

The data presented in Fig 2b further show that after more than 8 hours of treatment with mitomycin C the amount of radioactivity in the cold TCA soluble cell extracts decreased as compared with control cultures This delayed effect of mitomycin C was found also in cells which had been exposed to the antibiotic for 1 hour and then transferred to pure medium This finding seems to implicate that after prolonged incubation mitomycin C treatment suppressed incorporation of ^{3}H-TdR into DNA also by causing reduced uptake of ^{3}H TdR into the cold acid soluble cell pool

TABLE 2 Effect of Mitomycin C on Degradation of DNA

Mitomycin C (μg/ml)	Radioactivity in culture medium (cpm 10 $^{-3}$/ml) Hours of incubation				
	0	4	8	12	24
0 (control)	1 3	3 9	5 3	6 9	16 5
0 5		3 9	5 3	6 6	15 6
5 0		4 0	5 6	7 4	15 8

P 388 cells prelabelled with ^{3}H TdR were incubated in medium containing 10 $^{-5}$ M unlabelled TdR and mitomycin C in the concentrations indicated The amount of radioactivity in the culture medium was measured at zero time and after 4 8 12 and 24 hours of incubation

Effect of Mitomycin C on Degradation of DNA

Degradation of DNA was measured as release of radioactivity from cells which had been prelabelled with ^{3}H TdR Control cultures and cultures grown with 0 5 and 5 0 μg/ml of mitomycin C were analysed at time intervals during the first 24 hours after addition of the antibiotic From the data presented in Table 2 it can be seen that with both concentrations tested mitomycin C did not cause additional release of labelled material as compared with control cultures The

radioactivity found in the medium increased with the time of incubation in all cultures After 24 hours this fraction was calculated to about 5 per cent of the total radioactivity in the culture the remaining 95 per cent being found in the hot TCA soluble cell fraction

DISCUSSION

The present results clearly show that mitomycin C selectively and rapidly suppressed DNA synthesis in the P 388 cells (Figs 1 and 2a) Similar findings have been reported for other mammalian cells (*e g Magee & Miller* 1962, *Kontani* 1964) Apparently the observed inhibition first occurred on the DNA polymerization level since the uptake of ^{3}H TdR into the cold acid soluble cell pool remained unchanged for several hours (Fig 2b), and the formation of ^{3}H TTP was not affected after treatment for 2 5 hours (Table 1) This is compatible with the current view on the mechanism of mitomycin C action, rapid alkylation of cell DNA and blocked DNA replication at the alkylated sites (*Iyer & Szybalski* 1963, *Matsumoto & Lark* 1963) Obviously a direct effect on the DNA polymerase and/or a reduced production of DNA precursors other than TTP might also explain the present results Previous studies of the action of mitomycins in other organisms have shown that a polymerase preparation from treated HeLa cells effectively incorporated ^{3}H TdR into DNA (*Bach & Magee* 1962), and the size of the deoxyribotide pool was not reduced in treated bacteria (*Matsumoto et al* 1966 *Smith Kielland* 1966)

Extensive degradation of DNA was not an early effect of mitomycin C in the present cell line (Table 2) The early reports on the action of the antibiotic on mammalian cells stressing the importance of DNA breakdown (*Shatkin et al* 1962 *Kersten & Themann* 1962) were based on measurements performed after prolonged treatment DNA degradation associated with mitomycin C induced cell death take place also in P 388 cultures after more than 1 day of treatment (*Orstavik* 1972) However, the present negative findings cannot exclude a possible minor degradation connected with DNA excision repair as found in bacteria (review *Szybalski & Iyer* 1967), as DNA degradation products may be re utilized in repair synthesis (*Cleaver* 1967)

The data on DNA synthesis after mitomycin C had been removed might seem contradictory Colorimetric DNA assays (Fig 1) indicated that DNA synthesis was not permanently suppressed in cells which had been exposed to the antibiotic for 1 hour, whereas reversion of the initial inhibition could not be detected with the ^{3}H TdR labelling technique (Fig 2a) Since thymidine is not an essential DNA precursor, but represents an alternative source for TTP (*Cleaver* 1967), it is tempting to suggest that DNA synthesis proceeded after mitomycin C had been removed and that the treatment caused a specific suppression of the utilization of TdR This view is supported by the finding that the amount of radioactivity incorporated into cold TCA cell extracts decreased after more than 9 hours of incubation of these cells (Fig 2b)

The reduced uptake of ^{3}H TdR into the cold acid soluble cell pool was not further explored, but it might indicate an effect of the antibiotic on some mechanism for transport of TdR into the cells Interference with TdR kinase might be suspected, since phosphorylation may act as a regulating factor for uptake of TdR across mammalian cell membranes (*Schuster & Hare* 1971)

While it may be concluded from the present study that mitomycin C causes a rapid and strong suppression of DNA synthesis in the P 388 cells, the results are inconclusive regarding the reversibility of this effect, and also regarding DNA repair as a possible explanation of the reversible growth inhibition and giant cell formation induced by short treatment with the antibiotic (*Orstavik* 1972) These problems are currently being investigated

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PROTEIN A IN *STAPHYLOCOCCUS AUREUS* STRAINS OF HUMAN AND BOVINE ORIGIN

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Staphylococcus aureus strains from human infections and from bovine cases of acute and of chronic mastitis were studied regarding protein A content Human strains as well as bovine strains from acute mastitis showed high levels of protein A production in parallel with a high incidence of cell wall associated protein A In contrast, *S aureus* strains from bovine cases of chronic mastitis showed a significantly lower production of protein A Only about 50 per cent of these strains had detectable cell wall associated protein A Host-parasite relationship in staphylococcal infections might favour the presence of protein A in strain from acute infections In chronic bovine mastitis, however, the low levels of protein A detected in these strains seem to indicate that other factors play a more important role

The staphylococcal cell wall component protein A as defined by its reactivity with the Fc part of gamma globulin (*Forsgren & Sjöquist* 1966, *Kronvall & Williams* 1969), is detected in 90 (*Kronvall et al* 1971) to 99 per cent (*Forsgren* 1970) of human *staphylococcus aureus* strains isolated from human infections Protein A has also been found occasionally (0–2 per cent) in human *Staphylococcus epidermis* strains (*Forsgren* 1970, *Kronvall et al* 1971) but not in other bacteria (*Grov et al* 1970 *Kronvall et al* 1971) The role of protein A is not yet clear but may be a factor involved in pathogenicity (*Forsgren* 1971)

In the cow acute and chronic staphylococcal mastitis is a relatively common disease The immunoglobulins in the cow reacting in a non immune way with protein A are present in lower serum concentrations as compared to man (*Kronvall et al* 1970b, *Lind et al* 1970) A comparative investigation of the protein A content of *Staphylococcus aureus* strains isolated from man and from the cow seemed of interest The methods used were selected to detect cell wall associated protein A (*Winblad & Ericson*, to be published) as well as total protein A production (*Sjöquist & Stålenheim* 1969, *Forsgren* 1970) The results did not indicate any differences between the two species regarding strains from acute infections However, there were marked differences in the protein A content of strains isolated from acute and from chronic mastitis in the cow

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MATERIALS AND METHODS

Staphylococcal strains Two hundred and fifteen *Staphylococcus aureus* strains of human origin

were obtained from routine diagnostic work at the Clinical Microbiological Laboratory, the University Hospital Lund, Sweden, and represented consequtive cases from various sources, 102 strains from wounds and abscesses, 24 from septicemia 21 gastrointestinal, 38 urinary strains, and 30 strains from the nostrils of healthy carriers One hundred and twentyseven strains of *Staphylococcus aureus* of bovine origin were obtained from cases of acute (30) and chronic (97) mastitis diagnosed at the National Veterinary Institute, Stockholm The production of free coagulase was used as a taxonomic criterion for the designation of *Staphylococcus aureus* Seventy seven bovine strains of *Staphylococcus epidermidis* obtained from cow milk with elevated numbers of white blood cells were also included in the study All bovine strains were cultured on blood agar base supplemented with 5 8 per cent bovine blood The strains were subcultured once on the same medium and then stored at —20° C until use Bovine as well as human strains were propagated on CYT medium (Tris buffered 0 3 CY medium, *Novick* 1963, *Novick* & *Roth* 1968) or in CCY broth (*Arvidson et al* 1971) For the determination of cell wall associated protein A the strains were grown on CYT agar or on CCY medium (*Arvidson et al* 1971) containing 1 5 per cent agar

Qualitative determination of cell wall associated protein A A rapid slide method recently described by *Winblad* & *Ericson* (to be published) was used to detect cell wall associated protein A The strains were grown on CYT agar or CCY agar over night A colony was suspended in two drops of 3 per cent sensitized sheep red cells on a glass slide Almost all positive strains caused an agglutination to occur within seconds The slides were examined for up to 2 min before considered negative

Determination of the total protein A production by bacterial strains Bacterial strains were grown in 5 ml of CCY broth at 37° C over night Initially these over night cultures were read at 660 mμ to estimate the growth obtained Since the growths were essentially equal in the different cultures this step was later replaced by ocular inspection only After heating the cultures for one hour in boiling water to extract also cell wall associated protein A (*Forsgren* 1970) the cells were spun down and the supernatants subjected to further analysis The content of protein A in these supernatants was determined essentially as described by *Sjoquist* & *Stalenheim* (1969) The test samples were diluted in phosphate buffered saline containing 0 5 per cent human serum albumin (0 15 M NaCl 0 01 M phosphate pH 7 4 0 5 per cent HSA *SAP*) using Microtitre equipment (Cooke Engineering Company Alexandria Virginia 22314) Sensitized sheep red cells (0 25 per cent) were then added to each well including proper controls Finally a 1/500 dilution of a rabbit anti protein A antiserum was added and the sedimentation pattern read after a minimum of 3 hours. The addition of anti protein A antiserum made the test highly reproducible regardless of sheep red cells used and also increased the sensitivity The amount of protein A produced was expressed as the percentage of the amount produced by the reference Cowan I strain Extract of this staphylococcus as well as of the negative Wood 46 strain were included in every titration experiment as controls

Coagulase testing Human *S aureus* strains studied clotted human plasma diluted 1/5 in the tube test (*Blair et al* 1970) Bovine strains were similarly classified as *S aureus* when clotting rabbit plasma diluted 1/5

Phage typing Human strains of *Staphylococcus aureus* were phage typed using a standard set of human phages (*Blair* & *Williams* 1961) The bovine strains were tested using a set of phages described by *Davidson* (1961)

Enzyme production Human strains were analysed for lipase production on agar plates containing Tween 80 (*Sierra* 1957) Bovine *S aureus* strains were analysed for DNAse production (*Di Salvo* 1958) and egg yolk digestion (*Lundbeck* & *Tirunarayanan* 1966)

RESULTS

Qualitative Determination of Protein A

All 215 human 127 bovine *Staphylococcus aureus* strains and 77 bovine *Staphylococcus epidermidis* strains were tested for their capacity to agglutinate 3 per cent sensitized sheep red cell Such agglutination is mediated by protein A on the bacterial cell surface (*Winblad* & *Ericson* to be published) Out of 215 human *S aureus* strains grown on CYT agar medium 196 (91 2 per cent) agglutinated 3 per cent sensitized sheep red cells (Table 1) The overall incidence of 9 per cent for protein A negative strains corresponded well with a similar incidence in most of the strains when grouped according to type of infection However as many as four out of 21 human strains isolated from stool specimens were negative This incidence of 19 per cent negative strains differed from the incidence among the other human strains tested ($p < 0.05$)

All 30 bovine S aureus strains from cases of acute mastitis were positive in tests for cell wall associated protein A when grown on

TABLE 1 *Qualitative Determination of Cell Wall Associated Protein A in Human and Bovine Staphylococcal Strains*

		Medium used	Protein A Positive	Protein A Negative	Total number of strains
Human S aureus		CYT agar	196 (91 2 %)	19 (8 8 %)	215
Bovine S aureus	Acute mastitis	CCY agar	30	0	30
	Chronic mastitis	CYT agar	46 (47 5 %)	51 (52 5 %)	97
		CCY agar	58 (59 8 %)	39 (40 2 %)	
Bovine S epidermidis		CYT agar	0	48	77 (48)
		CCY agar	7	70	

CCY agar (Table 1) In contrast only 46 (47 5 per cent) or 58 (59 8 per cent) out of 97 bovine *S aureus* strains from chronic mastitis were positive when similarly tested from CYT agar or CCY agar cultures, respectively (Table 1) The difference in incidence between the two groups of bovine strains was significant ($p < 0\,0005$) as analysed by the chi square test. The human strains also differed significantly from bovine strains isolated from chronic mastitis ($p < 0\,0005$) There was no difference between the incidence of protein A detected in human strains and in strains from acute mastitis in the cow

No agglutination was noted when 48 bovine S epidermidis strains, grown on CYT-agar were tested However, four of the strains gave a weak agglutination when grown on CCY agar medium An additional 29 bovine *S epidermidis* strains grown on CCY agar were also studied As many as three of these agglutinated 3 per cent sensitized sheep red cells, one of them markedly so

Quantitative Determination of Protein A

The amount of protein A produced by 215 human and 127 bovine *Staphylococcus aureus* strains as well as 77 bovine *S epidermidis* strains was determined using a sensitized sheep red cell agglutination method All 215 human (100 per cent) and all except one (99 per cent) bovine *S aureus* strains produced detectable amounts of protein A In contrast,

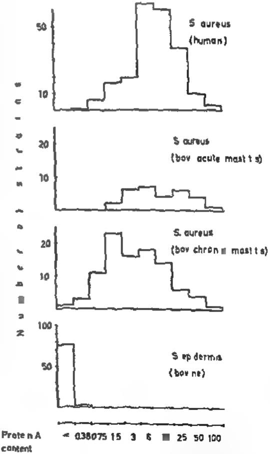

Fig 1 Distribution patterns of the results from quantitative determination of protein A in human and bovine staphylococcal strains The amount of protein A produced is expressed as the percentage of the amount produced by the reference Cowan I strain

only one out of 77 bovine *S epidermidis* strains was shown to produce protein A When the distribution of the values obtained was plotted, the differences between the groups became more apparent (Fig 1) The human *S aureus* strains as well as the bovine strains isolated from acute mastitis produced higher amounts of protein A as compared to the bovine *S aureus* strains from cases of chronic mastitis The mean values calculated from a base 2 logaritmic scale was 8 8 per cent of the Cowan I reference strain for the human strains and 3 8 per cent for the chronic bovine *S aureus* strains These two distribution patterns were statistically analyzed by comparing means and corresponding standard deviations as well as by chi square testing Using both methods the chronic bovine strain were shown to produce significantly less protein A ($p < 0\ 0005$) than the human strains The distribution of the acute bovine *S aureus* strains was also compared with the chronic bovine strains and these two groups were also shown to differ significantly ($p < 0\ 01$) No significant difference was noted between acute bovine strains and the human strains Among 77 bovine *S epidermidis* strains only one strain (1 3 per cent) showed a detectable level of protein A production. This strain was the same one giving a rapid and marked agglutination of 3 per cent sensitized sheep red cells in the slide method. The other six strains weakly positive in direct agglutination, did not show any detectable protein A production using the quantitative method

A comparison between the results obtained from the qualitative analysis of cell wall associated protein A and from determinations of total protein A production was also made. From the distribution of the human *S aureus* strains, a border line quantity of 4 16 per cent was calculated above which, statistically speaking the strains are expected to be positive in agglutination tests using 3 per cent sensitized sheep red cells From the distribution of the chronic bovine *S aureus* strains a border line value of 4 14 per cent was determined Although the quantitative distributions of the two materials were different as were the qualitative results the border line values calculated 4 16 and 4 14 per cent respectively, were strikingly similar This indicates a very good correlation between the

TABLE 2 *Presence of Cell Wall Associated Protein A in Human and Bovine S aureus Strains in Relation to Phage Groups*

A *Human phage groups*

		I	II	III	IV	Mixed	NT	Total
Protein A	present	63	23	52	1	6	51	196
	absent	4	6	2	1	0	6	19
Total		67	29	54	2	6	57	215

B *Bovine phage groups*

		I	II	III	IV	Mixed	NT	Total
Protein A	present	1	4	14	13	0	14	46
	absent	10	4	8	15	[illegible]	12	51
Total		11	8	22	28	2	26	97

The strains were grown on GYT agar medium over night and colonies were tested for agglutination of 3 per cent sensitized sheep red cells

two methods used. The chronic bovine *S. aureus* strains were also tested for cell wall associated protein A when grown on CCY agar. This substrate increases the sensitivity of the agglutination method to yield 59.8 per cent positive strains which gives a calculated border line value of 2.72 per cent of the Cowan I reference strain. A good substrate therefore seems to be of value in increasing the sensitivity of the qualitative method as has been pointed out previously (*Winblad & Ericson*, to be published). A direct comparison of slide agglutination and amount of protein A produced in individual human and bovine strains showed some variation. Only on a statistical basis was a very close correlation between the two methods made possible.

Protein A and Phage Groups

The phage groups of the 215 human *S. aureus* strains studied showed a pattern similar to 500 unselected *S. aureus* strains (*Hedström & Kronvall*, to be published). Absence of protein A on bacteria, as detected by the failure to agglutinate 3 per cent sensitized sheep red cells, showed a slightly uneven distribution among strains of various phage groups. As many as 6 out of 29 phage group II strains and 6 out of 57 NT strains were negative in the slide agglutination test. On the other hand, only 2 out of 54 group III and 4 out of 67 phage group I strains were negative in the slide test.

A Bovine serum

B Bovine colostrum

Fig. 2. Immunoelectrophoresis of bovine serum (Fig. 2A) and colostrum (Fig. 2B) after absorption with *S. aureus* strain Cowan I (precipitation line marked CI) and Wood 46 (precipitation line marked W). The immunoelectrophoretic slides have been photographed on top of each others to illustrate the change in the γ globulins taking place after absorption with Cowan I (CI) as compared to the normal pattern seen after absorption with Wood 46 (W). Rabbit anti bovine γ globulin serum was added to the antiserum trough.

The 97 bovine *S. aureus* strains were also phage typed and the phage groups of the individual strains were compared with presence or absence of protein A on the bacterial cell surface. The results obtained when strains grown on CYT-agar medium were used for the qualitative determination of protein A are shown in Table 2. A striking disproportion among the strains in phage group I is apparent with as many as ten strains out of eleven being negative as compared to an expected value of 5.7 strains. In phage group III the majority of the strains were positive. In the other phage groups the number of positive and negative strains were approximately equal.

Protein A in Relation to Lipase, DNAse and α-Toxin Production

The human *S. aureus* strains were tested for lipase (Tween 80 hydrolysis) and α toxin production and the results were compared with presence or absence of protein A in the same strains. There was no correlation between protein A on the cell wall and neither lipase nor α toxin production.

Thirty six bovine *S. aureus* strains from chronic mastitis were tested for lipase (egg yolk digestion) as well as DNAse production. When compared with qualitative protein A results when grown on CYT agar medium no significant correlation was obtained between protein A on the cell wall and lipase or DNAse production. However, when results from the more sensitive test for cell wall associated protein A using CCY-medium were analyzed lipase production did in fact correlate significantly ($p < 0.01$) with presence of protein A.

Absorption of Bovine Serum and Bovine Colostrum with Strain Cowan I and Wood 46

In order to evaluate the relative occurrence of protein A—reacting gamma glob-

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ulins in bovine colostrum, absorption with the protein A producing Cowan I strain as well as with the protein A negative Wood 46 strain was performed as described previously (*Lind & Mansa* 1968, *Kronvall et al* 1971) As is known from previous reports (*Lind et al* 1970) a smaller portion of bovine serum IgG is capable of reacting with protein A In Fig 2 this slowly migrating IgG fraction is illustrated Also *in colostrum only a minor* fraction of IgG present was found to be capable of reacting with protein A (Fig 2).

DISCUSSION

Previous attempts to correlate the presence or absence of staphylococcal protein A in infecting strains with distinct disease states have so far been largely unsuccessful (*Kronvall et al* 1971, *Nickerson et al* 1970, *Hedström & Kronvall*, to be published) In the studies presented here a difference in protein A content was noted in bovine *S aureus* strains isolated from cases of acute and of chronic mastitis The former strains resembled human *S aureus* strains in having a high protein A producing capacity and a high incidence of cell wall associated protein A In contrast, *S aureus* strains causing chronic mastitis in the cow, as diagnosed by elevated numbers of white cells in the milk (*Holmberg & Isaksson* 1970) showed significantly lower protein A production as well as a lower incidence of cell wall associated protein A It is noteworthy that strains from a particular human type of chronic condition predominantly caused by staphylococci, chronic osteomyelitis, do not show any decrease in the amount of protein A detected as compared to unselected human strains (*Hedstrom & Kronvall*, to be published)

The reasons for the difference found in the protein A content of strains from bovine acute and chronic mastitis are not yet clear The acute infection might depend on protein A as a pathogenicity factor as has been proposed for human infections (*Forsgren* 1970, *Kronvall et al* 1970a) In the human host one such mechanism involving complement activation might be operating With as much as 92 per cent of the normal human IgG being capable of reacting with protein A this staphylococcal product will give rise to complement fixing aggregates in the gamma globulin excess present In the cow, however, no reasonable mechanism for a protein A dependent increase in pathogenicity is evident Only slowly migrating γ globulins (Fig 1) can combine with protein A (*Lind et al* 1970) These gamma globulins are not capable of fixing complement (*Murphy et al* 1965, *Milstein & Feinstein* 1968) The gamma globulin present in bovine colostrum is mainly made up of actively excreted IgG-fast (*Pierce & Feinstein* 1965, *Aalund* 1968) As shown in Fig 2, a small but definite fraction of colostral IgG did combine with protein A This fraction might be a secreted IgG-slow or passively transferred IgG slow The interrelationships between bovine immunoglobulins and protein A and their role in acute versus chronic mastitis in the cow will be studied further

Two different methods have been used for the determination of protein A The first one is a modification of a microtitre agglutination test using sensitized sheep red cells according to *Sjöquist & Stålenheim* (1969) The other one is a recently described direct agglutination of sensitized sheep red cells by staphylococci The two methods were compared and shown to agree well When the bacteria were grown on agar containing high concentrations of nutrients the latter slide test could detect as little as 2 72 per cent of the amount of protein A produced by the Cowan I reference strain The two methods might however, give quite discordant individual results as has been described for many methicillin resistant strains (*Winblad & Ericson* to be published) Among *S epidermidis* strains analysed here a reverse situation seemed to exist Seven strains gave a positive reaction indicating presence of protein A on the bacterial cell surface whereas only one produced detectable levels when the total amount of protein A was determined This situation has indeed been predicted by *Forsgren* (1970)

It is therefore possible that the incidence of cell wall associated protein A in *S epidermidis* strains might be higher than previously found (*Forsgren* 1970, *Kronvall et al* 1971) This finding also raises the question whether

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STUDIES OF A LI-HAPTEN ISOLATED FROM CELL-WALLS OF THE ROUGH MUTANT *SALMONELLA TYPHIMURIUM* 395 MR10

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An O-haptenic material (LI fraction) was isolated from cytoplasmic and cell wall preparations of the rough mutant *Salmonella typhimurium* 395 MR10 The yield from the cell wall preparation virtually free from cytoplasmic contamination was approximately ten times higher than that from the cytoplasmic preparation The LI fraction obtained from cell walls was less contaminated with extraneous polysaccharide material After purification of the LI fraction with ribonuclease treatment and Sephadex G 100 gel chromatography structural studies using methylation analysis could be performed The hapten showed the same structural features as the O side-chains of the parent strain *S typhimurium* 395 MS The average number of repeating units in the hapten was 23 as compared to 11 in the O side-chain of the parent strain Molecular weight determination with Sepharose 6 B gel chromatography revealed that the LI fraction was polydisperse with a weight average of 26,300 and a number average of 21 800

The lipopolysaccharide (LPS) of *Salmonella* is a phosphorylated heteropolysaccharide covalently linked to a specific glucosamine containing lipid A (18) The polysaccharide contains the O antigenic determinants which provide the serological basis for the classification of *Salmonella* according to the Kauffmann White scheme (15) The polysaccharide has a complex structure the outermost portion of the O specific side-chain is in general built by repeating oligosaccharide units joined through glycosidic bonds The O specific side chain is then covalently linked to a basal core thought to be common to all *Salmonella*, containing *D* glucose, *D* galactose *N*-acetyl *D* glucosamine *L* glycero-*D* mannoheptose 2 keto-3 deoxyoctonate phosphate, and ethanolamine The basal core is in turn covalently linked to lipid A

The O specific side chain in *Salmonella typhimurium* is obtained by assembly and polymerization of tetrasaccharide repeating units from a lipid bound intermediate This side chain is then linked to the complete basal core Rough mutants of *Salmonella* synthesizing a more or less complete basal core therefore often accumulate the precursor of the O side chain (18) *Beckmann et al* (3) found a polysaccharide containing the sugars characteristic of the smooth parent

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strain in the supernatants (LI-fraction) after ultracentrifugation of the water phase after phenol water extraction of bacterial cells of a series of rough mutants By passive haemagglutination the authors also demonstrated that this fraction showed the antigenic specificity of the parent strain

Based on this discovery, a screening procedure for the detection of the LI fraction by gel precipitation when classifying rough mutants was developed (13) The antigen was prepared by heating a cell suspension of the rough mutants to 70° C for 30 minutes in a 0 02 M phosphate buffer at pH 7 In addition to the cell wall polysaccharide, these extracts evidently contained the LI-hapten, when present in the mutants

The present paper reports on a structural study of the LI hapten isolated from the rough mutant *S typhimurium* 395 MR 10 originating from the smooth parent strain *S typhimurium* 395 MS The hapten was isolated from cell-walls in higher yield than from a cytoplasmic fraction The main fraction purified by gel chromatography was subjected to a qualitative and quantitative chemical analysis and molecular weight determination The preparation was assayed further by passive haemagglutination inhibition

MATERIALS AND METHODS

... R10 was ... *rium* 395 ... bacterio ... *LPS)* of this mutant contains heptose as its dominant sugar component Abequose rhamnose mannose galactose and glucose monosaccharides characteristic of the LPS of the parent strain were found in minor amounts only (14) Based on the chemical composition of the LPS and the phage pattern obtained the strain was tentatively designated as a chemotype Rd mutant (17)

Isolation of the hapten from cell walls The strain was cultivated as described earlier (14) The bacteria were disintegrated in a cell homogenizer (W Braun Apparatenbau Melsungen Germany) and the LPS and hapten was extracted from the cell walls by hot phenol water (10) The hapten was recovered in the supernatant after ultracentrifugation of the water phase of the phenol water extract at 105,000 × g for 4 hrs The supernatant was lyophilized and named crude LI fraction Treatment of the crude LI fraction by pancreatic ribonuclease (Sigma), (enzyme/crude LI fraction = 1/100 w/w), for 24 hrs at 37° C, followed by dialysis against distilled water at +4° C, removed contaminating RNA since no specific absorption at 260 nm was observed

Further separation of the LI fraction was obtained by gel chromatography on a Sephadex G 100 column (Pharmacia Fine Chemicals Uppsala Sweden) B24 × 110 cm, equilibrated with 0 05 M phosphate buffer, pH 7 2 and 0 5 M NaCl The column was eluted with the 0 05 phosphate buffer containing 0 5 M NaCl in order to avoid absorption of the LI hapten to the gel Flow rate was 10 9 ml per hour and the fraction volume 5 0 ml The carbohydrate content of the fractions was estimated by the phenol sulphuric acid method (22)

Sugar analysis of the hapten The procedure ... bed by Sawardeker ... identified by their retention times and ... spectra (6)

Methylation analysis of the hapten The procedure used (4, 5) involves exhaustive methylation of the polysaccharide its hydrolysis into a mixture of monomeric methylated sugars reduction, acetylation and the separation identification and quantitative estimation of the components in the mixture The position of glycosidic linkages in the polysaccharide corresponds to the position of unmethylated hydroxyl groups in the partially methylated sugar derivatives

Molecular weight of the hapten The molecular weight of the isolated hapten was estimated by Sepharose 6 B gel filtration The number average and weight average molecular weight was determined according to *Granath and Kvist* (7)

Passive haemagglutination inhibition The technique described by *Beckmann et al* (2) was used Serial twofold dilutions of the hapten were prepared in phosphate buffered saline, pH 7 2 Antisera diluted to contain 4 to 8 haemagglutinating units were added to each dilution and the mixture was incubated at 37° C for 30 min Sheep red blood cells sensitized with phenol water extracted wild type *S typhimurium* LT2 LPS were added and the mixture was incubated at 37° C for 30 min and at room temperature for 3 hrs The lowest concentration of LPS showing complete macroscopic inhibition of agglutination was determined The homologous LPS/antiserum systems represented the *Salmonella* 0 factor antisera 04 05 and

O1? (15), and rough mutants of chemotypes Ra Rb Rc and Rd (17)

RESULTS

Anacker et al (1) and *Rudbach et al* (20) isolated from *E coli* a polysaccharide 'native protoplasmic polysaccharide', which apparently was of cytoplasmic origin This polysaccharide was isolated from the supernatant after trichloracetic acid (TCA) treatment of the protoplasmic fraction of disintegrated bacteria In our initial attempts to isolate the hapten for structural analysis this method was used The polysaccharide fractions isolated after TCA treatment of the cytoplasmic fraction from *S typhimurium* 395 MR 10 their subsequent treatment with pancreatic ribonuclease and amylase and after gel filtration on Sephadex G 100 on sugar analysis yielded the data given in Table 1 Approximately 80 per cent of the sugars detected in fraction II could derive from an O specific side-chain Since no heptose was detected it was probable that the material represented a haptenic fraction Assuming a regular structure of the side chain with Abe Man Rha Gal ratios of 1 1 1 1 (10) it can be seen that only half of the amount of *D* mannose detected could originate from the O side chain The origin of the other half of *D* mannose is uncertain but it might come from a mannan The average number of repeating units in the side chain is calculated from the methylation analysis data by determining the ratio chain *D* mannose (4 6 di *O* methyl *D* mannose) chain terminating *D* mannose (2 4 6 tri *O* methyl *D* mannose) Due to the presence of the extra *D* mannose no determination of the chain length could be done In addition the analysis of fraction III revealed a large proportion of *D* glucose probably originating from a glucan The possible presence of a glucan as well as a mannan in the fractions thus made the cytoplasmic fraction unsuitable as a source of the O specific hapten for structural studies In addition the LI-hapten obtained represented only 0 01 per cent of the bacterial dry weight

LI hapten isolated from cell walls Cell walls were obtained from washed *S typhimurium* 395 MR 10 bacteria after disintegration with glass beads and subsequent differential centrifugation The cell wall fraction appeared to be virtually free from contaminating cytoplasmic material since no specific absorption at 260 nm could be observed The supernatant (crude LI fraction) obtained after ultracentrifugation of the dialysed water phase of hot phenol water extracted cell walls displayed however absorption at 260 nm Treatment with ribonuclease followed by dialysis removed most of the contaminating nucleic acids since less than 1 per cent of ribose could be detected in the quantitative sugar analysis The Abe Rha Man Gal ratios of the crude LI fraction were approximately 0 4 0 9 1 0 1 1 (Table 2) Considerable amounts of heptose 45 per cent of detected sugars were also found in the crude LI fraction The heptose most probably originated from 395 MR 10 LPS which did not sediment at 105 000 × g In passive haemagglutination inhibition the concentrations of

TABLE 1 *Analysis of Sephadex G 100 Fractions on Haptenic Material Isolated from the Cytoplasm of S typhimurium 395 MR10*

Fraction	Per cent polysacch	Mole per cent of detected sugars: Abe	Rha	Man	Gal	Glu	Per cent O specific sugars
Crude		9	14	48	16	13	
I	93	11	16	48	16	9	64
II	70	13	21	40	18	8	80
III	42	1	2	14	2	81	8

TABLE 2 *Analysis of Haptenic Material Isolated from Cell Walls of* S typhimurium *395 MR 10 after Purification on a Sephadex G 100 Column*

Fraction	Weight (mg)	Per cent polysacch	Mole per cent of detected sugars					
			Abe	Rha	Man	Gal	Glu	Hep
Crude LI	38.1	25	5	13	14	15	8	45
Purified LI	15.3	28	14	25	23	29	9	ND

the crude LI-fractions required for inhibition of the following homologous LPS/O factor sera systems were 04 16 μg/ml, 05, 0.5 μg/ml and 012_2, 128 μg/ml The homologous 395 MR 10 LPS/antiserum system was inhibited by 8 μg/ml of the crude LI-fraction This is in accordance with the demonstration of heptose in the sugar analysis of this fraction and the assumption that all of the 395 MR 10 LPS did not sediment in the ultracentrifuge Upon gel chromatography on a Sephadex G-100 column two included fractions were obtained The major fraction represented 40 per cent of the material applied on the column The Abe Rha Man Gal ratios were approximately 0.6 1.0 0.9 1.2 (Table 2) No heptose could be detected in this fraction Assuming that the LI hapten is a direct precursor to the O side chain all of the monosaccharides detected could represent such a hapten In passive haemagglutination inhibition only 04, 05, and 012_2 LPS/antiserum systems were inhibited This fraction was therefore assumed to represent the LI hapten and was subjected to structural studies using methylation analysis The LI hapten represented approximately 0.1 per cent of the bacterial dry weight The second fraction

TABLE 3 *Methyl Ethers from the Hydrolysates of the Methylated LI Fraction from 395 MR 10 and the LPS from the Parent Strain* S typhimurium *395 MS**

Sugars	T†	Molar proportion° LI fraction	395 MS
2 4–di *O* methyl abequose	0.32	9.2	7.0
2, 3–di *O*–methyl *L* rhamnose	0.98	21.4	21.4
2, 3 4 6–tetra *O*–methyl–*D* glucose	1.00	0.9	1.8
2 3 4, 6–tetra–*O* methyl *D* galactose	1.25	ND§	1.3
2, 4 6–tri *O*–methyl *D* mannose	2.08	0.9	2.1
2, 4, 6–tri–*O*–methyl–*D*–galactose	2.26	20.6	20.9
4, 6–di *O*–methyl–*D* mannose	3.29	19.6	21.2
2 6–di–*O*–methyl *D* galactose	3.62	0.9	1.8
3, 6–di–*O* methyl *D* galactose	4.30	ND§	1.1
2 4–di–*O* methyl *D* glucose	5.10	ND§	1.1

* The data from the methylation analysis of the parent strain *S typhimurium* 395 MS (10) are included for comparison

† Retention times (T) of the corresponding alditol acetates on the ECNSS M column relative to 1, 5–di–*O* acetyl–tetra–*O*–methyl *D*–glucitol

° Since a considerable proportion of 2 4–di *O*–methyl–abequose was lost during the methylation analysis the per cent of the methylated sugars is given relative to that of 2 3–di *O* methyl *L*–rhamnose assumed to be the same as the mole per cent of *L*–rhamnose in the original lipopolysaccharide The figure for the methyl ether of *L* rhamnose in the LI fraction is adjusted to the value found for the ether in the wild type strain *S typhimurium* 395 MS The percentages of the other methylated sugars are given relative to the 2 3–di *O* methyl–*L* rhamnose derivative

§ ND = not detected

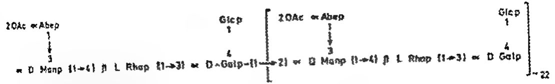

Fig 1 Proposed structure of the O side-chain of the LI hapten from *S typhimurium* 395 MS The anomeric nature of the linkages is according to (8) and (9)

contained almost exclusively KDO (identified as a thiobarbituric acid positive material) but the amounts obtained were not sufficient for further analyses

Structural analysis of LI hapten The methyl ethers obtained in the methylation analysis of the LI fraction were the same as those obtained in the structural investigation of the LPS of *S typhimurium* strains 395 MS and LT 2 (10 11 and Table 3) The molar percentages of abequose, L rhamnose D mannose D galactose and D glucose calculated from the methylation analysis and assuming that the molar percentage of L rhamnose is the same as in the original lipopolysaccharide are 10 25 24 25 1 The agreement between these values and those obtained from the sugar analysis of the hapten is good with the exception of *D* glucose There is no obvious reason to account for the observed difference

Based on the methylation analysis of the hapten together with the information from the structural analysis on the LPS from the parent strain 395 MS the following structure was proposed (Fig 1) From the percentage of 2 4, 6 tri O methyl *D* Mannose (representing the nonreducing end of the side-chain), and of 4 6-di O methyl *D* Mannose (representing chain *D* Mannose) the average number of repeating units in the hapten side-chain was estimated to be about 23 The average number of repeating units in the O side-chain of the parent strain 395 MS was 11 (10)

The amount of polysaccharide was insufficient for an analysis of the the content of O-acetyl groups The data obtained in passive haemagglutination where 0 25 μg hapten/ml inhibited the homologous 05/*S typhimurium* LT 2 LPS system whereas 128 μg/ml were required for a deacetylated preparation justify the presence of O-acetyl groups linked to C 2 of abequose in Fig 1

The presence of 0 9 per cent of 2, 3, 4 6-tetra *O* methyl *D* glucose and 2, 6-di-*O*-methyl *D* galactose indicates that the *D* galactose residue in every 23rd repeating unit carries a *D* glucose residue linked to C-4 The corresponding figure in 395 MS was that the *D* galactose of every 12th repeating unit carried a *D* glucose substituent (10)

None of the minor components obtained in the methylation analysis of the LPS from strain 395 MS (Table 3) were found in the LI hapten Structural studies of the LPS from rough mutants of *S typhimurium* LT 2 (18) have revealed that these components most probably derived from the core polysaccharide Their virtual absence as well as the lack of methylated heptose residues in the chromatogram is a further proof that the

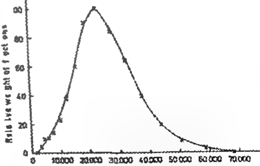

Fig 2 Molecular weight distribution curve of the purified LI hapten on a Sepharose 6 B column calibrated with dextrans of known molecular weights (7)

methyl ethers found derive from a LI hapten and not from a LPS fraction

Molecular weight of the LI-hapten The molecular weight of the purified LI-hapten was estimated by gel filtration on a Sepharose 6 B column (Fig 2) The figures given are estimated by comparison of the data obtained when dextrans of known molecular weight are applied on the same column The weight-average was estimated to 26,300 and the number-average to 21,800

DISCUSSION

The precipitation patterns obtained with extracts from Gram negative bacteria display a great variation depending on the method used for the preparation of the antigen and on the variable antibody response to these antigens in the rabbit (12) In the earlier studies the O specific LPS antigen of several *Salmonella* strains could be shown to give a precipitation line near the antigen well, using homologous sera The detection of a second line, apparently also showing O specific characteristics, was of special significance since it indicated the presence of a smooth-specific antigen in rough mutants of Salmonella (13) These antigens have been demonstrated in passive haemagglutination by *Beckmann et al* (3) and were believed to represent a cytoplasmic hapten (LI-fraction) Since the antigens giving the two O specific lines could be separated by ultracentrifugation it was concluded that the line near the antigen well represented the high molecular weight LPS and that the other O specific line represented the hapten

The fact that both precipitates were present in the gel precipitation system indicates that some of the determinants of the complete antigen were not present in the hapten Otherwise one would assume that the antibodies should be quantitatively precipitated by the hapten *Anacker et al* (1) using purified haptenic material arrived at the same conclusion Our initial attempts to isolate and purify the LI hapten from the cytoplasmic fraction were unsuccessful A haptenic fraction which in passive haemagglutination inhibition showed O4, O5, and O12 reactivity was isolated, but the qualitative sugar analysis showed unexpectedly high values of *D* glucose and *D* mannose (Table 1) The extra amounts of these sugars made the preparation unsuitable for a structural study by methylation analysis In addition the total amount of LI hapten isolated from the protoplasmic fraction was low, less than 0 01 per cent of the cell dry weight

Kent and Osborn (16) recently studied the nature of the O specific hapten They isolated the hapten by phenol water extraction from rough mutants of *S typhimurium* and purified it by ultracentrifugation, ethanol precipitation and DEAE cellulose column chromatography Their data showed the hapten to be firmly bound to particulate cell envelope fractions but not to be exposed to the cell surface

Our data obtained when we succeeded in preparing a LI-fraction from cell wall preparations, with only small amounts of contaminating cytoplasmic material (judged by the lack of specific absorption at 260 nm) support those obtained by *Kent and Osborn* (16) Thus the hapten should accumulate in the envelope in rough mutants A probable location, though not proved is on the outside of the cytoplasmic membrane Support for this theory is obtained by the demonstration that treatment of the cell walls of rough mutants with EDTA and lysozyme (16 19) which removes the outer membrane and peptidoglycan in gram negative bacteria exposes the LI hapten Extraction of the bacteria, or isolated cell-walls with phenol water cleaves the pyrophosphate linkage between the hapten and the antigen carrier lipid (ACL) to which the hapten is linked (16)

The average number of repeating units in the isolated hapten was 23 compared to 11 in the smooth parent strain *S typhimurium* 395 MS The reason for the difference in the average number of repeating units between these two preparations might be that since the defect in the synthesis of the core results in a LPS lacking the site to which the O side

chains normally attach, the LI-hapten accumulates in the cell envelope Under these circumstances the repeating unit polymerase might accomplish an elongation of the O side chain So far, however, nothing is known about the regulation of the activity of the repeating unit polymerase

The approximate molecular weight of an O side chain with 23 repeating units where all abequose residues carry an O acetyl group, every 23rd D galactose residue carries a D glucose substituent and with a terminal D galactosyl 1 phosphate residue is 15,000 The distribution of molecular weights as determined by gel chromatography indicates that a variation in the number of repeating units per hapten molecule may exist (Fig 2) Since the number average is approximately 50 per cent higher than the value expected from the data of the methylation analysis, one could assume that a fraction of the hapten molecules exists as aggregates, di tri or, possibly, tetra meres It is however, also concievable that the comparison of the hapten to dextrans of known molecular weight gives a too high value for the hapten, since the branched dextran molecule is more compact than the hapten with its proposed linear structure Further studies are necessary to settle this point

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EVALUATION OF SOME EXTRACTION METHODS FOR THE PREPARATION OF BACTERIAL LIPOPOLYSACCHARIDES *FOR STRUCTURAL ANALYSIS*

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The extraction of lipopolysaccharides (LPS) from smooth strains of *Salmonella typhimurium* for structural studies using methylation analysis is preferably done by hot phenol water method on a cell wall preparation obtained by mechanical disintegration of γ irradiated bacteria The LPS obtained by this method was less contaminated than LPS extracted by hot phenol water from acetone dried bacteria or bacteria pretreated with 1 per cent formaldehyde LPS for structural analysis from rough mutants is, due to its lipophilic character, preferably extracted with a mixture of phenol chloroform and petroleum ether The presence of the precursor of the O side chain, LI hapten, in the different LPS and supernatant fractions was assessed by gel precipitation tests The LPS forms a line near the antigen well whereas the low molecular weight hapten forms a line near the antiserum well The hapten was not formed in excess in the smooth strain *S typhimurium* LT2 In the rough mutant studied, *S typhimurium* SL 733 O-antigenic material was found in both the LPS preparation (probably as a covalently linked O side chain) and the supernatant (as a hapten)

Several methods have been used for the isolation of structures carrying the O antigenic specificity from Gram negative bacteria (see 20, 22) Most of them have been developed empirically Depending on the goal of the investigation the whole O antigenic complex (= endotoxin) or its more or less degraded components have been extracted

The O-antigen is a complex built of lipopolysaccharide (LPS), proteins and phospholipids found in the outer membrane of Gram negative bacteria The polysaccharide side-chains (O side chains) of the LPS carry the immunological specificity of the respective O antigens (20) For a structural analysis of these O antigens it is therefore preferable to extract only the LPS or even better, only the polysaccharide side-chains, from the outer membrane of the bacteria Present information suggests that the LPS is bound in the

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Abbreviations Hep = heptose, Glc = *D* glucose Gal = *D*-galactose Man = *D* mannose, Rha = *L*-rhamnose Abe = abequose, GNAc = *N* acetyl *D*-glucosamine, Rib = *D* ribose, P = phosphate Etn = etanolamine KDO = 2-keto-3-deoxyoctonate PhW = extraction with hot phenol water PCP = extraction with a mixture of phenol chloroform and petroleum ether

cell-wall primarily by physical forces, *i e* hydrophobic, ionic, or both (27)

One of the most commonly used extraction methods is the phenol-water procedure of *Palmer and Gerlough* (23) as modified by the Westphal group (26) The bacteria are treated with a mixture of 90 per cent phenol and water (1 1 v/v) at 68° C for 5 minutes After cooling of the mixture the water phase is isolated It contains LPS, nucleic acids and other polysaccharides In our studies on LPS from smooth and rough strains from *Salmonella typhimurium* by a qualitative and quantitative sugar analysis using gas liquid chromatography of peracetylated alditol acetates (16), the LPS preparations obtained by phenol-water extraction from dried whole bacteria were found unsuitable In addition to peaks identified as alditol acetates of the sugar constituents a number of peaks containing non-carbohydrate material were detected Therefore, some procedures used for the isolation of LPS were evaluated in order to obtain preparations more suited for quantitative and structural studies

Lipopolysaccharides were extracted by the phenol water procedure from (i) dried, whole bacteria, (ii) bacteria pretreated with 1 0 per cent formaldehyde, and (iii) cell-wall preparations obtained after mechanical disintegration of the bacteria with glass beads and subsequent differential centrifugations and washings The different LPS-preparations were assessed by a qualitative and quantitative sugar analysis using gas liquid chromatography (24) The preparations were further analysed by ultraviolet absorption spectrometry for an estimation of the amount of contaminating nucleic acids, and gel precipitin test for detection of the LI-hapten (O side-chain precursor) (15) Lipopolysaccharides obtained from cell-wall preparations were found to be the superior material for structural studies In addition, the LPS preparations obtained from rough mutants either by phenol-water extraction of cell wall preparations or phenol, chloroform and petroleum ether (PCP) extraction of dried, whole cells (9), were compared

MATERIALS AND METHODS

Bacterial strains The characteristics of the smooth strain *Salmonella typhimurium* LT2 and its rough mutant *S typhimurium* SL 733, strain st/22 of *Yamamoto and Anderson* (28), have been described earlier (13, 18) The rough mutant *S typhimurium* 395 MR10 originates from the smooth strain *S typhimurium* 395 MS (16)

Cultivation and extraction procedures The bacteria were grown and harvested as described earlier (16) Each strain was re isolated from a single colony and tested for nutritional characteristics serological specificity and phage sensitivity before and after cultivation (18)

The hot phenol water extraction procedure (26) for the extraction of LPS and a haptenic fraction was applied onto (i) acetone dried bacteria according to *Sutherland* (25), (ii) bacteria pretreated with formaldehyde (1 per cent final concentration) over night at +4° C, and (iii) a bacterial cell wall preparation obtained by disintegrating washed bacteria in a Braun disintegrator (B Braun, Apparatenbau, Melsungen, Germany)

The bacteria were killed by irradiation with ^{60}Co γ rays (12000 rad/min for 1 h) before disintegration The bacteria (7 5 g, wet weight) were then suspended in distilled water (30 ml) and shaken together with glass beads (30 g 0 17-0 18 mm) Tributyl phosphate (0 2 ml) was added as an antifoam The bacteria were completely disintegrated, as judged by the fact that no intact bacteria could be observed when the preparations were examined by phase microscopy after 3 min of shaking at maximum speed The temperature was kept below +5° C by a stream of carbon dioxide After disintegration, the glass beads were removed by filtration through a coarse glass filter Initial separation of cell walls from the cell debris was effected by centrifugation at 10,000 × g for 20 min in an IEC PR2 refrigerated centrifuge at +4° C The cell walls collected as a loosely packed layer on the pellet surface The wall preparation was washed off carefully It was subsequently washed, in the cold twice with 0 1 M phosphate buffer (pH 7 0), once with 1 M sodium chloride and once with distilled water The yield of cell walls was about 10 per cent of the bacteria (dry weights) The cell wall preparation did not display any specific absorption at 260 nm, indicating the absence of nucleic acids or purine pyrimidine compounds in the wall preparations

The aqueous phase after phenol water extraction, was extracted five times with ethyl ether to remove phenol and then dialysed against distilled water No monosaccharides could be detected in the dialysis water The water phase was then lyophilized The yield of LPS referred to as crude LPS was about 200 mg per g of cell walls (dry

ABLE 1 *Sugar Composition of Lipopolysaccharides and Supernatant Fractions from* Salmonella typhimurium *LT2, SL 733 and 395 MR10*

	Extraction method*		Ribose†	Per cent of PS in preparation§	Mole per cent of detected sugars°							Number of repeating units per O side chain
					Hep	Glc	Gal	Man	Rha	Abe	GNac	
	Aceton killed bacteria	LPS	19	25	11	14	24	17	16	16	2	3 0
	PhW	Sup	37	12	10	17	23	17	16	16	1	3 3
	Formaldehyde treated bacteria,	LPS	3	40	7	14	23	18	18	18	2	5 1
	PhW	Sup	[illegible]	44	10	18	18	15	14	14	10	2 9
	Cell walls,	LPS	<1	40	10	11	26	16	17	17	3	3 3
	PhW	Sup	7	28	9	11	28	20	14	14	1	3 8
	Cell walls	LPS	<1	30	29	31	29	3	3	4	ND	0 2
	PhW	Sup	26	25	[illegible]	5	38	29	9	[illegible]	ND	3 8
	Acetone killed bacteria,	LPS	4	20	35	29	32	<1	<1	<1	ND	<0 1
	PCP	Sup	–	Not analysed	–	–	–	–	–	–	–	–
R10	Cell walls	I PS	2	19	70	5	9	5	4	4	ND	0 1
	PhW	Sup	12	25	41	8	14	13	12	12	ND	0 6
		LI hapten	ND	28	ND	9	29	20	21	21	ND	–

* PhW phenol water extraction, PCP = phenol, chloroform, petroleum ether extractions LPS refers to the pellet obtained after 2 cycles of ultra centrifugation of the crude LPS fraction at 105 000 × g Sup refers to the supernatant fraction obtained after ultracentrifugation of the crude LPS fraction at 105 000 × g

† Mole per cent of ribose detected compared to total amount of monosaccharides detected

§ PS = polysaccharide The amount of polysaccharide in each preparation was estimated with D xylose as internal standard in the sugar analysis

° The responce factor for heptose was 1 6 and for N acetyl [illegible] glucosamine 2 0 ND = not detected <1 means that the monosaccharide was found to be present but in concentrations ranging between 0 1 and 0 9 per cent of detected sugars

weight) for smooth bacteria and 50 to 125 mg for rough bacteria.

The LPS preparation (1 mg/ml in distilled water) was checked for specific absorption at 260 nm. The LPS preparations were then subjected to two cycles of 1 ... 4 h ... to ... obt...

... *analysis* The procedure was based on the method described by *Sawardeker et al* (24) and modified according to *Hellerqvist & Lindberg* (11) The carbohydrate content of each preparation was estimated with D xylose as an internal standard The different sugars were identified by their retention times and mass spectra (10)

Gel precipitation tests These were done as described earlier (14) Antiserum was prepared by injecting 0 2 mg antigen (cell wall preparation, dry weight) of *S typhimurium* 395 MS (O antigens 4, 5, 12$_2$) intravenously into rabbits twice a week for 9 weeks

RESULTS

The results of the qualitative and quantitative sugar analyses obtained on LPS preparations extracted from the smooth strain *Salmonella typhimurium* LT 2 and the rough mutant *S typhimurium* SL 733 are compiled in Table 1

Strain LT2 After phenol-water extraction, the water phase of the different preparations was subjected to ultracentrifugation at

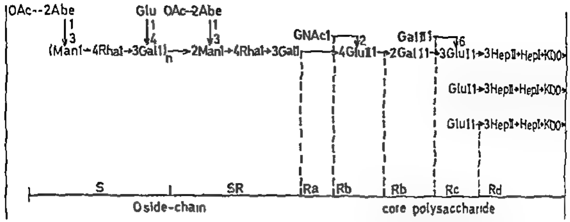

Fig 1 Proposed structure of the polysaccharide side chain of *Salmonella typhimurium* LT2 based on (11) and (13)

105,000 × g By this procedure the LPS-fraction with an average molecular weight of several millions is sedimented whereas contaminants, such as the O antigenic precursor (LI-hapten) and nucleic acids, remain in the supernatant

Structural analyses on the LPS of strain LT 2, as well as on LPS from rough mutants originating from it, have revealed that ribose is not a structural component of these LPS (11, 13) The amount of ribose detected, probably deriving from RNA, could therefore be used as a criterion for the purity of the LPS preparation The data obtained revealed that the LPS extracted from a cell wall preparation contained < 1 per cent of ribose compared to 3 per cent and 19 per cent, respectively, when the LPS were extracted from formaldehyde-treated or acetone killed and dried bacteria The sugar analyses on the supernatants obtained after ultracentrifugation clearly demonstrated the purification obtained by this procedure since the amounts of ribose in these preparations were more than twice as high as in the sedimented LPS-preparations

The carbohydrate content of each preparation, estimated with xylose as an internal standard in the sugar analyses, varied between 25 per cent and 50 per cent

Structural analyses have revealed that the O specific side chain in strain LT 2 is built by repeating tetrasaccharide units of abequose, mannose, rhamnose and galactose (13) The amounts of mannose, rhamnose and abequose (corrected for the destruction during acid hydrolysis (11)) in the LPS preparations were approximately equimolar The amounts of galactose were, however, higher Besides being a structural component of the O specific side-chain, galactose is also present in the core polysaccharide where it, according to present knowledge is equimolar to heptose By deducting from the total galactose value the contribution from the core polysaccharide galactose is found to be approximately equimolar to abequose, mannose and rhamnose The corresponding 1 1 1 1 ratios were also found in the supernatant fractions tested

An approximate estimation of the average number of repeating units per O side chain can be obtained by calculating the ratio mole per cent rhamnose + mole per cent mannose per mole per cent heptose The approximate number in these LPS preparations was found to be from 3 to 5 repeating units

Strain SL 733 The mutation leading to roughness in this strain is supposed to affect the transfer of *N* acetyl *D* glucosamine to the core polysaccharide (18) As a consequence the O side chain precursor (LI hapten) can not attach the core but accumulates in the cell envelope (3 15 17, 19)

The LPS extracted from the cell wall preparation by phenol water did not contain detectable amounts of ribose, whereas the

PCP extracted preparation contained 4 per cent Structural analysis of a PCP-extracted preparation revealed the approximate molar ratios of heptose glucose galactose to be 1 1 1 (11), which also was found with this PCP preparation The phenol-water extracted LPS preparation also yielded the same ratios In addition, both LPS preparations contained small but significant amounts of O specific sugars Two reasons may account for this, either a fraction of the LI hapten sediments with the LPS at 105,000 × g, or the defect leading to roughness is incomplete, "leaky" In this connection "leakage" implies that a *N*-acetyl-*D* glucosamine transferring enzyme with low activity able to transfer some glucosamine residues to the core [redacted] synthesized The result will be that the completed core polysaccharides are capped by O-specific side-chains

Only the phenol-water extracted cell-walls yielded a supernatant fraction in amounts sufficient for a sugar analysis This revealed very high amounts of galactose and mannose in addition [redacted] core sugar and sugar characteristic of the O side-chain

Gel precipitation tests The precipitation patterns obtained with the LPS and supernatant preparations studied above as antigens, and an antiserum, containing O factor 4, 5 and 12, antibodies, are shown in Fig 2a-2d As a comparison the LPS and supernatant of the rough mutant *S typhimurium* 395 MR 10 and a purified LI hapten from the latter with an average molecular weight of 26,300 were included (19)

The precipitation lines near the antigen wells in Fig 2a show reaction of identity They apparently represent the precipitate of the high molecular weight LPS and homologous antibodies A second line is demonstrated most clearly by the supernatant fraction in antigen well no 4 The origin of the second line is shown in Fig 2b This line is formed with the supernatant fraction from *S typhimurium* 395 MR 10, from which the low molecular weight hapten was isolated and with the crude 395 MR 10 fraction and [redacted] therefore identified as the hapten line

Only a weak "hapten line" was displayed by the supernatant from the LT 2 cell-wall preparation, which means that the LI-hapten was not formed in excess by the smooth parent strain

The LPS extracted by phenol-water from a cell-wall preparation of strain SL 733 (Fig 2c) displayed a weak line showing identity with the "hapten line" displayed by the supernatant from the same preparation The PCP-extracted LPS fraction of SL 733 did not display any precipitation line in the concentrations tested The PCP supernatant did, however, display a weak precipitation line showing identity with the "hapten line"

The identification of the precipitation line near the antiserum well as a 'hapten line" was supported by the observation that the purified 395 MR 10 LI-hapten (Fig 2d) yielded a line showing reaction of identity with the other preparations from the same strain, and with the supernatant fraction of the PhW-extract from LT 2 cell walls It was not possible to demonstrate reaction of identity with the "LPS line" situated near the antigen well A similar result has been reported by *Anacker et al* (2)

DISCUSSION

The development of a methylation analysis, using gas liquid chromatography (5) and mass spectrometry (4) for the qualitative and quantitative analysis of the alditol acetates of methylated sugars, suitable for structural analysis of LPS called for the preparation of pure starting materials Several extraction methods, such as the trichloracetic acid methods (7), the diethylene glycol method (21), extraction with aqueous pyridine or glycol (6), DMSO (1) and aqueous ether (8) all have in common that, besides the LPS, other structural components of the cell wall such as phospholipid, lipoprotein and proteins are co extracted often in the form of a complex Therefore these preparations have to be subjected to several purification steps, sometimes including treatment under alkaline or acid conditions, which may de-

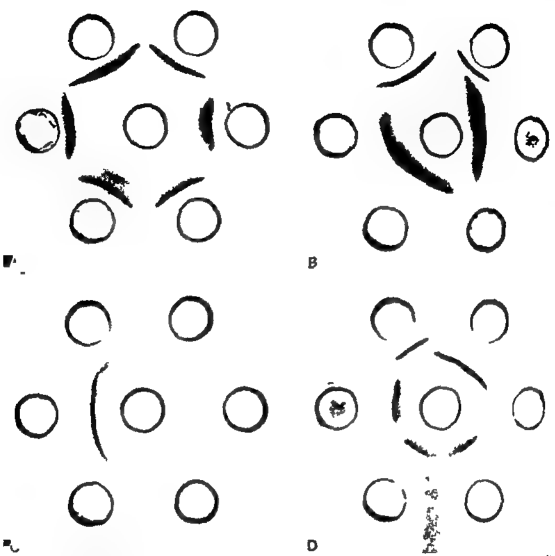

Fig 2 Gel precipitation patterns obtained with different antigen preparations from strains LT2, SL 733, and 395 MR10 and antiserum containing O factors 4 5 and 12 Designation of antigens, crude LPS dialysed water phase after PhW or PCP extraction, LPS, pellet obtained after 2 washings of crude LPS at 105 000 × g Sup, supernatant 1 after ultracentrifugation of crude LPS at 105 000 × g The antigens were placed clockwise in 6 wells (1–6) around a central antiserum well (Ab) according to the drawing The antigen concentration in each well was 100 μg/ml

6		1
5	Ab	2
4		3

Fig 2A 1 LT2 acetone PhW, LPS, 2 LT2, formaldehyde, PhW LPS, 3 LT2, formaldehyde, PhW, crude LPS 4 LT2, formaldehyde, PhW sup, 5 LT2, acetone, PhW, sup, 6 LT2, acetone PhW, crude LPS

Fig 2B 1 LT2, cell walls, PhW, LPS, 2 395 MR10, cell walls, PhW, crude LPS 3 395 MR10, cell walls, PhW, LPS, 4 395 MR10, cell walls, PhW, sup, 5 LT2, cell walls PhW, sup, 6 LT2, cell walls, PhW, crude LPS

Fig 2C 1 SL 733 cell walls, PhW, LPS, 2 SL 733, acetone, PCP LPS, 3 SL 733, acetone, PCP, crude LPS, 4 SL 733 acetone, PCP, sup, 5 SL 733, cell walls, PhW, sup, 6 SL 733, cell walls, PhW, crude LPS

Fig 2D 1 395 MR10, cell walls, PhW, purified hapten, 2 LT2, cell walls, PhW, sup, 3 395 MR10, cell walls PhW, ribonuclease treated supernatant fraction 4 395 MR10, cell walls, PhW sup, 5 395 MR10, cell walls, PhW, crude LPS 6 LT2, cell walls, PhW crude LPS

stroy important structural features in the polysaccharide The phenol water method (26) is unique among the commonly used extraction methods since it preferentially extracts the LPS and capsular antigens When the method is applied on whole bacteria as is commonly done, certain cytoplasmic constituents such as nucleic acids and glucans are found in the water phase together with the LPS The O side chain precursor (LI hapten) present in certain rough mutants is also found in the water phase The LPS is freed from these contaminants by high speed ultracentrifugation at 105 000 × g but a complete separation is seldom achieved Bacterial cell wall preparations can therefore be considered as a superior starting material for the phenol water extraction of LPS compared to whole bacteria

Because of the risk of generating potentially hazardous aerosols during the disintegration procedure the Salmonella bacteria had to be killed It was found that γ irradiation was the most satisfactory procedure Comparative analyses by methylation of LPS extracted from irradiated and non irradiated *S typhimurium* LT 2 bacteria revealed that with the dosage used, the irradiation did not impose any structural changes in the polysaccharide side chain The killing of the bacteria with disinfectants (1 per cent formaldehyde 0 3 per cent β propiolactone) or heat made them resistant to disintegration Disintegration by freezepressing was also tried The cell wall preparations were however more contaminated by cytoplasmic constituents when this method was used as compared to shaking with glass beads even when additional washings were included in the procedure for isolating the walls The contamination was detected both as higher absorption in the UV region and the presence of ribose in the polysaccharide analysis This difference is probably explained by the solubilization of cytoplasm obtained when the disintegration is performed in liquid as is the case when the bacteria are shaken with glass beads

The ribose content (Table 1) revealed that the LPS obtained from the cell wall preparation of *S typhimurium* LT 2 was less contaminated with nucleic acids than the LPS preparation obtained by extraction of acetone dried or formaldehyde pretreated whole bacteria Despite several washes of the cell wall preparation in order to remove contaminating material some probably membrane components were still attached to the wall preparation This was evident from the finding of 7 per cent of ribose in the supernatant fraction The formaldehyde pretreated bacteria also yielded a LPS preparation with low nucleic acid content

Ultracentrifugation was used as a purification step for the separation of the LI hapten fraction from the LPS The results of both the sugar analysis and the gel precipitation patterns strongly favour the view that the O side chain precursor is not formed in excess in the smooth LT 2 strain

The approximate number of repeating units in the O side chain of strain LT 2 was estimated by methylation analysis to be about 9 (13) Using the ratio *D* mannose + *L*-rhamnose/heptose, assuming that each O side chain is linked to a core with two heptose residues the approximate number was estimated to be 3–5 (Table 1) Most core side chains in strain LT 2 seem to be capped by O side chains as revealed by methylation analysis (unpublished data) The observed discrepancy can be due to the fact that each core side chain contains more than two heptose residues (*Hellerqvist & Lindberg* unpublished observations)

When the sugar composition of the LPS extracted from rough mutants was studied (15 16 18) small amounts of the sugars characteristic for the O specific side chain were found Two reasons may account for this either the LPS preparation studied was contaminated with the LI hapten or the mutation leading to roughness was incomplete leaky resulting in the synthesis of a greatly reduced number of O side chains The sugar analysis of strain SL 733 revealed the presence of O specific sugars in the LPS preparation from cell walls

The PCP extraction method is supposed

to extract only the lipophilic core LPS whereas a LPS with O side-chains is hydrophilic and as such not extracted (9) The sugar analysis of the PCP-extracted LPS revealed, however, small amounts of O specific sugars which could mean that LPS molecules with a few O side-chains are extracted No "LPS line" was found in the gel precipitation test A possible explanation of that is that the amount of O-specific material in the concentration of LPS tested (1000 μg/ml) was too low to yield a visible precipitate Attempts to increase the LPS concentration failed due to low solubility of the SL 733 LPS

Of the two methods tested for the extraction of LPS from rough mutants, the PCP-method (9) was superior to the phenol-water method applied onto a cell-wall preparation This is due to the fact that LPS from revertants to the parent smooth form are not extracted and, if present in the rough mutant, the amount of O-specific sugars is too small to disturb the structural analysis of the core polysaccharide

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SEMLIKI FOREST VIRUS GLYCOPROTEINS AND CANAVANINE

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By means of carbohydrate labelling two virus specific glycoproteins were revealed in BHK21 cells infected with Semliki Forest virus a non structural protein (NSP68) and a structural protein (VP53) In addition, a third virus induced, carbohydrate-containing compound (G20) was found, which is probably a glycopeptide In the presence of canavanine under conditions in which viral RNA synthesis continued but no virus was produced the glycosylation of both VP53 and NSP68 was reduced, whereas the formation of G20 was totally inhibited The possibility that NSP68 is a precursor for VP53 is suggested by the results of pulse-chase experiments During the chase period the radioactivity of VP53 increased, while that of NSP68 decreased correspondingly With canavanine present, the amount of radioactivity in NSP68 and VP53 remained unchanged during the period of observation strongly supporting the above hypothesis

In the cytoplasm of cells infected with group A arboviruses several virus specific non-structural proteins and two structural proteins have been identified by polyacrylamide gel electrophoresis (*Hay et al* 1968, *Scheele & Pfefferkorn* 1969, *Strauss et al* 1969 *Ranki* 1972) The smaller lysine-rich structural protein, which has a mol wt 32 000–34 000 (*Acheson & Tamm* 1970b *Simons & Kääriäinen* 1970, *Kennedy & Burke* 1972) is associated with the viral nucleocapsid (*Friedman* 1968, *Strauss et al* 1968, *Kääriäinen et al* 1969, *Acheson & Tamm* 1970a) and the larger structural protein, with a mol wt 50 000–55 000, (*Acheson & Tamm* 1970a, *Simons & Kääriäinen* 1970 *Kennedy & Burke* 1972) with the viral envelope (*Strauss et al* 1968, *Kääriäinen et al* 1969 The envelope protein of Sindbis virus is a glycoprotein containing glucosamine mannose, galactose, fucose and sialic acid (*Burge & Strauss* 1970, *Strauss et al* 1970) The same sugars have also been found in the Semliki Forest virus (SFV) envelope protein (*Renkonen*, to be published)

Canavanine, an amino acid analogue of arginine, inhibits the synthesis of SFV (*Ranki & Kääriäinen* 1969) If added to infected cultures in the middle of the growth cycle, it allows virus RNA and nucleocapsids to be synthesized but effectively reduces the amount of envelope protein produced (*Ranki & Kääriäinen* 1970 *Ranki* 1972)

In the present study advantage was taken of the canavanine inhibition Two glycoproteins were found in the infected cells one of which is the envelope protein, here designated VP53, and the other a non structural protein, NSP68 The degree of glycosylation

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of VP53 and NSP68 was clearly reduced in the presence of canavanine and the synthesis of the virus induced glycopeptide, G20, detected during the normal infection was totally inhibited

Kinetic analyses in the presence and absence of the inhibitor suggest that SFV has two envelope proteins one of which is a cleavage product of NSP68

MATERIALS AND METHODS

Virus and Cells

The origin of Semliki Forest virus its cultivation infectivity and haemagglutination assays have been described previously (*Kaariainen et al* 1969 *Ranki & Kääriäinen* 1969) BHK21 cells clone WI 2 were cultivated in BHK21 medium (Gibco manual Grand Island NY) supplemented with 10 per cent calf serum and 10 per cent tryptose phosphate broth (*Ranki & Kaariainen* 1969)

Preparation of Cytoplasmic Extracts

Complete monolayers mainly in Petri dishes (5 cm diameter) or Falcon plastic bottles (260 ml) were infected with SFV at a multiplicity of infection of [redacted] as described previously (*Ranki* 1972) Eagle's minimum essential medium usually deficient in arginine supplemented with 0 2 per cent bovine serum albumin and containing actinomycin D (1 µg/ml) was used as growth medium This medium was replaced by one containing canavanine (200 µg/ml) (Calbiochem California) but no arginine usually 3 hr after infection The infected cultures were pulsed as follows ^{35}S methionine (14 C/mM The Radiochemical Centre Amersham England) was given in the growth medium deficient in methionine ^{14}C amino acids from Chlorella protein hydrolysate (59 mC/mAtom carbon Amersham) were given together with ^{3}H-glucosamine (3 6 C/mM New England Nuclear (NEN) Boston Mass) in a medium containing one tenth of the normal amino acid concentration Other sugar precursors ^{3}H mannose (230 µC/mM Amersham) ^{3}H-galactose (0 5 mC/mM Amersham) ^{3}H fucose (4 3 C/mM NEN) were given in the growth medium L-amino acids ^{3}H mixture was purchased from NEN The pulse length varied from 3 min to 3 hr The preparation of the cytoplasmic extract after swelling the cells in RSB (0.01 M Tris pH 7 4 0 01 M KCl 0 0015 M $MgCl_2$) douncing them and pelleting the nuclei has been described previously (*Ranki* 1972) The nuclear pellet was treated with 2 per cent Nonidet P40 (NP40) (Shell Chemical Co) in RSB and the supernatant after repelleting the nuclei was collected and added to the former post nuclear supernatant. The trichloracetic acid precipitable radioactivity was determined as described previously (*Ranki & Kääriäinen* 1969) The protein content of the cytoplasmic extract was measured according to *Lowry et al* (1951)

SDS Polyacrylamide Gel Electrophoresis

(5 or 7 5 per cent acrylamide recrystallized from chloroform according to *Loening* (1967) with 0 2 per cent bisacrylamide) was performed according to *Weber & Osborn* (1969) as described previously (*Ranki* 1972) The electrophoresis was carried out at pH 7 in 0 1 M sodium phosphate buffer containing 0 1 per cent sodium dodecyl sulphate (SDS) and 0 1 per cent 2 mercaptoethanol, at 3 V/cm and room temperature The cytoplasmic extracts for analysis were treated with 1 per cent SDS and 1 per cent mercaptoethanol for 2 min at 90° C and dialysed overnight against the electrophoresis buffer The radioactivity of the 2 mm gel slices was determined in NCS toluene as described by *Caliguiri et al* (1971) The cytoplasmic extracts pulsed for 3 min with ^{35}S methionine were treated with pancreatic ribonuclease (200 µg/ml) (Worthington Biochemical Corp Freehold New Jersey) for 10 min to breakdown the amino acid and tRNA complexes before adding SDS and mercaptoethanol

Pronase Treatment

The cytoplasmic extract labelled with ^{14}C amino acids and ^{3}H-glucosamine was treated with pronase (100 µg/ml) (B grade Calbiochem California) for 6 hr at 37° C The pronase solution was preincubated at 37° C for one hr to minimize glycosylase activity

Lipid Extraction

Lipids were extracted from the cells after labelling with ^{3}H glucosamine or oleic 9 10 H^3 (N) acid (10 C/mM NEN) and different lipid fractions were isolated as described elsewhere (*Renkonen et al* 1972)

RESULTS

Synthesis of SFV Proteins in BHK21 Cells

A short pulse of ^{35}S methionine was given to BHK21 cells infected with SFV, 5 hr after infection Polyacrylamide gel electrophoresis in the presence of SDS and mercaptoethanol showed that about 60 per cent of the radioactivity migrated in the position of the non structural proteins mol wts 110 000 95 000 and 68 000 (Fig 1A) (*Hay*

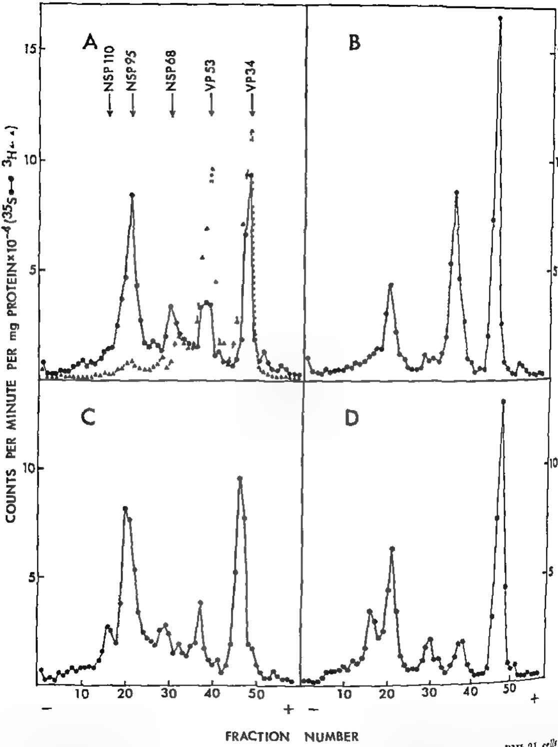


Fig 1 Polyacrylamide gel electrophoresis (5 per cent gel) of SFV proteins synthesized in BHK21 cells 5 hr after infection Pulse with ^{35}S methionine (●——●) (100 μC/Falcon bottle) for 3 min and chase with an excess of unlabelled methionine for 2 min (A) further chase for 45 min *(B) Pulse (C)* and chase (D) in the presence of canavanine Samples A and C were treated with ribonuclease (200 μg ml) for 10 min at 37° C before treating with SDS (▲——▲) cytoplasmic extract from *infected* cells pulsed with ^{3}H amino acids for 1 hr starting 5 hr after infection

TABLE 1 SFV *Proteins Synthesized in BHK21 Cells after Pulsing with* ^{35}S *Methionine for 3 Min and Chasing for 45 Min*

lasmic t	NSP110	%	NSP95	%	NSP68	%	VP53	%	VP34	%	Total incorporation
	Counts per min/mg protein										
rol											
lse	79300	7.5	233000	22	153800	15	130900	12	208650	20	1.06×10^6
ase	56930	5.9	125300	13	47070	5	248400	26	292700	31	0.94×10^6
avan ne											
lse	101050	8.6	307700	26	127350	11	120300	10	263250	22	1.17×10^6
ase	119000	12	192020	20	84300	8.9	78460	8.2	292050	31	0.95×10^6

The distribution of radioactivity in the cytoplasmic extracts after analysis on 5 per cent polyacrylamide gels is presented. The pulse was started 5 hr after infection.

TABLE 2 SFV *Proteins Synthesized in BHK21 Cells after Pulsing with* ^{14}C *Amino Acids*

Cytoplasmic extract	Per cent of the total counts NSP95	NSP68	VP53	VP34
3 min pulse	15	19	16	16
chase for 10 min	12	19	16	18
chase for 20 min	9	15	19	22
chase for 30 min	9	13	23	22
chase for 60 min	9	8	29	19
continuous pulse for 60 min	3	11	30	27

The distribution of radioactivity in the cytoplasmic extract after analysis on 5 per cent gels is presented. Cells were pulsed 5 hr after infection with ^{14}C-amino acids (10 μC/dish) for 3 min and chased with a 5-fold excess of unlabelled amino acids.

et al. 1968, *Ranki* 1972). After incubation in the presence of a 10 fold excess of unlabelled methionine for 45 min (chase), the distribution of the radioactivity was markedly altered in the gel. The viral structural proteins, the envelope protein and the nucleocapsid protein dominated. For convenience the non-structural proteins are marked as NSP110, NSP95 and NSP68 and the structural proteins correspondingly VP53 and VP34.

The pulse chase experiments were performed in the presence of canavanine under conditions in which viral RNA synthesis continues but virus production is inhibited (*Ranki & Kaariainen* 1970). After the 3 min pulse the distribution of radioactivity was similar to that obtained in the uninhibited controls (Fig. 1C), the only difference being the accentuation of NSP110 (*Strauss et al.* 1969). The chase period had less effect on the ^{35}S activity distribution in the inhibited cells than it did in the controls, although the peaks became better defined (Fig. 1D). NSP68, which clearly decreased during the chase period in the absence of canavanine, was unaltered. At the same time the increase in VP53 seemed to be inhibited (Table 1).

Both in the presence and absence of canavanine the proportion of VP34 increased during the chase period and that of NSP95 decreased. This experiment has been repeated with ^{14}C amino acids (Table 2). The results were basically the same, only the increase in VP34 was less marked.

In Fig. 1A the distribution of radioactivity after a continuous pulse for 1 hr is seen. The predominance of the structural proteins is apparent. The pattern observed remained more or less constant independent of the phase of the growth cycle provided host protein synthesis was sufficiently inhibited (i.e. 3 hr or more after infection).

The Glycosylation of the Cytoplasmic Proteins

Both control and canavanine treated infected cells were pulsed with ^3H-glucosamine and ^{14}C amino acids and the cytoplasmic proteins analyzed on 7.5 per cent polyacryl-

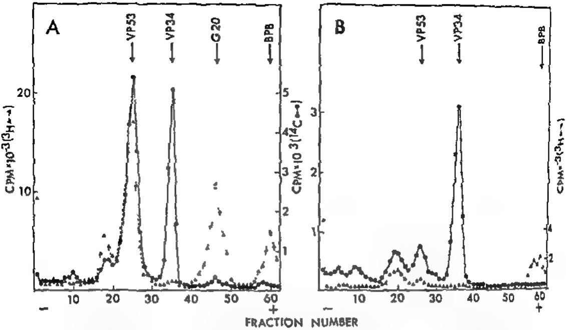


Fig 2 Glycosylation of SFV proteins synthesized in BHK21 cells Infected cultures on Petri dishes were pulsed with ^{14}C amino acids (1 μC/dish) (●——●) and ^{3}H glucosamine (20 μC/dish) (▲ ▲) from 5 to 8 hr after infection, some as controls (A) and others after treatment with canavanine beginning 3 hr after infection (B) The analysis of the cytoplasmic extracts on 7 5 per cent gels is presented The migrations of gangliosides, extracted from infected cells after labelling with ^{3}H glucosamine as above as well as of BHK21 cell lipids, labelled with ^{3}H oleic acid, and ^{14}C UDP glucose were studied in parallel on separate gels The ganglioside peak was in fraction 59 the BHK lipids in fraction 62 and UDP glucose in 64 BPB = bromphenol blue band

TABLE 3 *The Distribution of the Carbohydrate Label in the Cytoplasmic Proteins of Cells Infected with SFV*

Glycoprotein	Glucosamine		Mannose		Galactose		Fucose	
	Contr	Can	Contr	Can	Contr	Can	Contr	Can
NSP68	5 8	1 1	4 9	1 2	5 0	0 3	3 7	–
VP53	3 0	0 79	1 3	0 75	2 1	0 34	1 5	–
G20	30	–	4 8		28	–	28	–

^{3}H glucosamine (20μC/dish) was given together with ^{14}C amino acids (1 μC/dish) for 3 hr from 5 to 8 hr after infection in the presence and absence of canavanine ^{3}H mannose (100 μC/dish), ^{3}H galactose (100 μC/dish) and ^{3}H fucose (50 μC/dish) were given under identical conditions Cytoplasmic extracts were analyzed on 7 5 per cent gels The table presents the ratios of ^{3}H carbohydrate to the ^{14}C-amino acids in each glycoprotein peak

Estimates of the quantities of carbohydrates metabolized to amino acids during the 3 hr period were made using the radioactivity detected in the position of the nucleocapsid as a standard It was calculated that 8 per cent of the glucosamine 20 per cent of the mannose and 30 per cent of the galactose counts were not in their carbohydrate form, and these corrections were made before calculating the ratios presented in the table

amide gels Glucosamine label was detected in VP53, as well as in NSP68, and in a smaller component with an apparent mol wt of 20 000 (G20) (Fig 2A) The ratio of glucosamine to protein counts was twice as high in NSP68 as in VP53 In G20 this ratio

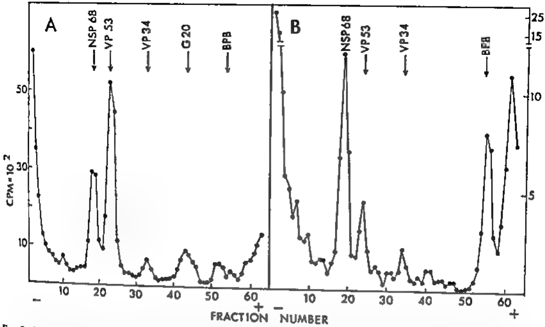


Fig 3 Polyacrylamide gel electrophoresis (7 5 per cent gel) of SFV proteins in BHK21 cells labelled with ^{3}H mannose (100 µC/dish) from 5 to 8 hr post infection Untreated controls (A), canavanine added 3 hr post infection (B) BPB – bromphenol blue Approximately 20 per cent of the mannose counts come from amino acids derived from mannose (see explanation of Table 3)

was about 10 times higher than in VP53 (Table 3) In the presence of canavanine the distribution of the glucosamine label clearly differed from that in the control (Fig 2B) The total incorporation was significantly smaller and the degree of glycosylation of both NSP68 and VP53 was reduced (Table 3) Similar results were obtained when mannose and galactose were used as labelling material (Fig 3, Table 3) Fucose, known to be a terminal sugar in cellular glycoproteins (*Ginsburg & Neufeld* 1969) was not incorporated into the viral proteins in the presence of canavanine indicating that the carbohydrate chains are probably left incomplete (Table 3) The most striking difference, however, was the total absence of G20 in the presence of canavanine Nor could it be detected in uninfected cells, which suggested it to be virus induced It was found to contain all the carbohydrates used Glucosamine, galactose mannose and fucose (Fig 2, Fig 3, Table 3)

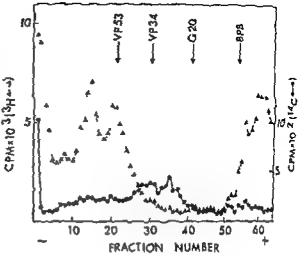
Characterization of the G20 Component

The possibility that the fast migrating carbohydrate containing component consisted of glycolipids was excluded as follows Lipids were extracted from glucosamine labelled infected cells and the resulting gangliosides, as well as ^{3}H oleic acid labelled BHK21 cell lipids, were treated like the protein samples with SDS and analyzed separately on polyacrylamide gels The lipids migrated together with the bromphenol blue (BPB) band and were very clearly separated from G20 Again after labelling the cells with ^{14}C acetate, a negligible amount of radioactivity as compared to the huge quantity in the lipids, was found in G20, indicating that it really did not contain a significant proportion of newly synthesized lipids

Finally, UDP glucose ^{14}C was used as a representative nucleotide sugar, it clearly migrated ahead of the buffer front

The ^{14}C amino acids co-migrating with G20 indicated the possibility of a glycopeptide This assumption was supported by the

Fig 4 Polyacrylamide gel electrophoresis (7.5 per cent gel) of the cytoplasmic extract from infected cells, labelled with ^{14}C amino acids and ^{3}H glucosamine from 5 to 8 hr post infection as for Fig 2, after pronase treatment (100 µg/ml for 6 hr at 37° C). The untreated sample was simultaneously electrophoresed and the migration of VP53, VP34 as well as of G20 are indicated by arrows ●——● ^{14}C amino acids ▲ ▲ ^{3}H glucosamine



results obtained after pronase treatment. Cytoplasmic extract from infected cells labelled with ^{3}H glucosamine and ^{14}C amino acids as above, was treated with pronase. Subsequent analysis on polyacrylamide gels showed that the G20 peak had disappeared (Fig 4). When the G20 component was eluted from the gel before treating it with pronase a similar result was obtained after re electrophoresis.

DISCUSSION

The function of the non structural proteins identified by polyacrylamide gel electrophoresis in arbo A virus infected cells is not known but increasing evidence has accumulated pointing to precursor product relationships between the larger non structural and the structural proteins (*Strauss et al* 1969, *Burrell et al* 1970, *Igarashi* 1970, *Scheele & Pfefferkorn* 1970, *Pfefferkorn & Boyle* 1972). The results presented here support this hypothesis.

The largest well discernible non structural protein (NSP110) also found in Sindbis virus infected cells (*Strauss et al* 1969), was accentuated by canavanine. This protein could well be a precursor for several proteins as has recently been suggested by *Schlesinger et al* (1972).

Scheele & Pfefferkorn (1970), using a Sindbis ts mutant defective in nucleocapsid synthesis, demonstrated that NSP95 was the principal polypeptide in the infected cells at the nonpermissive temperature. They interpreted this as meaning that NSP95 is a precursor of VP34, the nucleocapsid protein. Our results do not contradict this hypothesis. But it has to be pointed out that after a short pulse with ^{14}C amino acids about three quarters of the total VP34 was already formed. During the chase period a gradual increase in the labelled VP34 (up to a quarter of the initial quantity) was observed. However, pulse chase experiments with ^{35}S methionine gave a larger increase of VP34 during the chase period. In both cases a concomitant decrease in NSP95 was observed. It is not yet known whether the rapidly labelled VP34 (*Friedman* 1969, *Scheele & Pfefferkorn* 1970) is identical with the slowly labelled portion.

A precursor product relationship between the two glycoproteins NSP68 and VP53 is in accordance with our data and seems highly probable. Canavanine prevented the increase of VP53 and corresponding decrease of NSP68 otherwise seen during the chase period while the other changes in the distribution of radioactivity were almost unaffected. About half of the VP53 was labelled already after the short pulse whereas the other half accumulated slowly. With canavanine present the synthesis of the

rapidly labelled VP53 seemed to be unaltered, but there was no substantial addition during the chase

On the basis of our results the synthesis of Semliki Forest virus envelope proteins could be as follows One of the envelope proteins is synthesized rapidly (*e g* as a primary gene product as suggested by *Friedman* 1969) The other, inhibited by canavanine, is a cleavage product from the non structural glycoprotein NSP68 The recently reported separation of Sindbis virus envelope proteins into two different glycoproteins by discontinuous disc electrophoresis (*Schlesinger et al* 1972) is in agreement with the above scheme

The seemingly different effects of canavanine on the synthesis of VP34 and VP53 may well be related to the reduced glycosylation of NSP68 in the presence of the drug

Labelling with radioactive carbohydrates revealed a new component G20 It contains at least glucosamine mannose, galactose and fucose The sugars are apparently bound to a small amount of newly formed protein, a peptide, as shown by ^{14}C amino acid labelling and sensitivity to pronase

This G20 glycopeptide is probably virus specific for the following reasons i) We were not able to find it in uninfected cells neither was it detected by *Gahmberg* (1971) who studied the glycoproteins of BHK21 cells ii) This compound is formed under conditions in which host protein synthesis is severely inhibited (*Strauss et al* 1969, *Kääriäinen*, unpublished results) iii) Its formation is totally inhibited in the presence of canavanine, given to the cells 3 hr after virus infection However, the genetic capacity of SFV RNA is insufficient to specify the carbohydrate sequence by coding the several sugar transferases required (*Ginsburg & Neufeld* 1969) Preliminary evidence shows that the carbohydrates of Sindbis virus envelope proteins are at least partially of host origin (*Burge & Huang* 1970, *Grimes & Burge* 1971) On the other hand the glycoprotein of vaccinia virus envelope is totally under the control of the viral genome (*Garon & Moss* 1971, *Moss et al* 1971)

The function of the glycopeptide component, G20, is not known for the present One possibility among others is that it could be a split product from one of the virus glyco proteins, *e g* NSP68

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BRIEF REPORTS

REASSOCIATION OF REDUCED IgM TO COVALENTLY BOUND 8S AND 17S MOLECULES

Bjarte G Solheim

When IgM is reduced with 0 1–0 2 M 2 mercapto ethanol in pH 8 0 buffered saline all disulfide bonds between the μ and light chains are reduced, and the protein falls apart into its subunits (6, 14, 15) Several authors have reported that removal of the reducing agent results in reassociation of the reduced IgM subunits to macroglobulin molecules (1 7 8 9) and a return of antibody activity (3 5) If oxygen is present together with the mer captan formation of mixed disulphides between the SH groups of the protein and the reducing agent would be expected reducing the yield of reasso ciated material This could be one explanation as to why the extent of reassociation varies consider ably Formation of mixed disulphides in IgM through a d sulphide interchange reaction has been descr bed by *Schrohenloher* (11)

The purpose of the present work was to study the influence of the protein concentration and the effect of oxygen on the degree of formation of mixed disulphides during reassociation of IgM

Materials and Methods

A monoclonal macroglobulin (Mø) was purified by euglobul n precipitation and subsequent gel filtration on Sephadex G 200 (4) The protein was pure IgM as judged by immunoelectrophoresis aga nst rabb t-antihuman serum Protein concen trations were determ ned from optical density measurements at 280 mμ using an extinction coeff cient $E_{1\,cm}^{1\%}$ 12 or by the Folin Ciocalteu method as described by *Miller and Metzger* (6)

The prote n was dissolved and reactions per formed in sal ne made 0 015 M in Tris (Hydroxy methyl) aminomethane buffer of pH 8 0

Radiolabelling with ^{125}I was performed by the electrochemical method of *Rosa et al* (10), as modified by *Solheim et al* (14) The macroblobulin was labelled to a specific activity of 47 μC/mg and had a mean iodine content of 7 atoms per mole cule The radiochemical purity was tested and the samples counted as described previously (4, 12) Iodoacetamide-1-^{14}C (Batch 13) was delivered by The Radiochemical Centre, Amersham, England

Reduction and reassociation The reactions were performed at 4° C in the dark Reduction was effected by dialysing not more than 2 ml protein solution against 1 litre 0 2 M 2 mercaptoethanol (ME) in pH 8 0 buffered saline for 24 hours For the reassociation experiments 2 ml of the reduced protein was dialysed against 1 litre portions of pH 8 0 buffered saline three different procedures being applied

1) Oxygen was bubbled through the buffer prior to and during the 48 hour dialysing period
2) The protein was dialysed for 4 hours against each of 1 to 5 changes of buffer which was in equilibrium with the atmosphere before the dialysis started The bottles were kept closed with a rubber stopper during dialysis the last dialysing period was 48 hours long
3) The protein was first dialysed for 4 hours against each of 1 to 4 changes of degassed (oxygen free) buffer in a closed system under a nitrogen atmosphere and subsequently for 48 hours against one shift of buffer which was in equilibrium with the atmosphere before the dialysis started

Alkylation was effected by dialysing not more than 2 ml reduced protein solution against 1 litre 0 001 M iodoacetamide which was dissolved im mediately before use in oxygen free saline made 0 03 M in Tris buffer of pH 8 0 dialysis was car ried out in the dark at 4° C for 24 hours under nitrogen atmosphere The alkylated protein was subsequently extensively dialysed against saline When labelled with iodoacetamide-1-^{14}C, 0 1 mC of recrystallized radioactive material was dissolved in 1 litre saline with add tion of cold iodoaceta

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mide to a final concentration of 0 001 M (14) Alkylation was then performed as described

Density gradient ultracentrifugation of the reassociated preparations was performed on 10–40 per cent sucrose density gradients, and the various fractions were assayed for ^{125}I activity. IgM reassociated under these conditions revealed two major peaks upon density gradient ultracentrifugation (4) The fast sedimenting peak sedimented slightly slower than native IgM (4), and was termed the 17 S fraction (9) The slow sedimenting peak termed the 8 S fraction, sedimented at a rate characteristic for a reduced and alkylated subunit with intact intrasubunit disulphide bonds and slightly faster than IgG (4, 9, 13)

Gel filtration on Sephadex G 200 was performed by upward flow elution of 2 ml samples on 2 5 × 100 cm Pharmacia columns equilibrated with

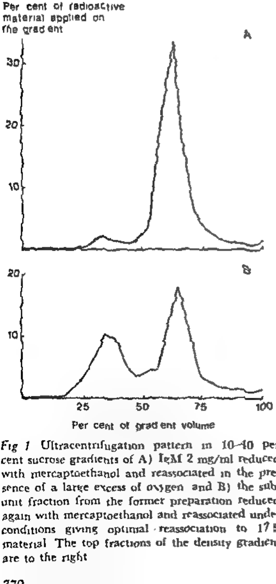

Fig 1 Ultracentrifugation pattern in 10–40 per cent sucrose gradients of A) IgM 2 mg/ml reduced with mercaptoethanol and reassociated in the presence of a large excess of oxygen and B) the subunit fraction from the former preparation reduced again with mercaptoethanol and reassociated under conditions giving optimal reassociation to 17 S material The top fractions of the density gradient are to the right

0 002 M borate buffer of pH 8 0 made 0 32 M in NaCl, or with 0 1 N propionic acid made 6 M in urea (13)

Results and Discussion

Unreduced IgM was included in all reassociation and alkylation experiments. These IgM preparations sedimented identically to native IgM on density gradient ultracentrifugation and showed the same elution pattern as the native protein on gel filtration With attempted alkylation with iodoacetamide-1-^{14}C less than one molecule iodoacetamide bound per IgM molecule

Reassociation procedure 1 When IgM (2 mg/ml) was reduced with 0 2 M ME and reassociated by dialysis against buffer with excess of oxygen, only a small amount of protein reassociated to fast sedimenting material (Fig 1A) With attempted alkylation of the subunit preparation with iodoacetamide-1-^{14}C less than 0 2 molecules iodoacetamide bound per subunit, indicating that the SH groups were modified by formation of mixed disulphides or by oxidation to sulphinic or sulphonic acid The experimental conditions excluded the formation of peroxides and thus reduced oxidation to sulphinic and sulphonic acids When the 8 S fraction was reduced once more with 0 2 M ME and alkylated 8 8 molecules of iodoacetamide bound per subunit, while 10 molecules bound per subunit upon reduction of intact IgM When the fraction reduced for the second time was reassociated by procedure 3 instead of alkylated, about 40 per cent of the subunits reassociated to fast sedimenting material (Fig 1 B)

IgM reduced and reassociated by procedure 1 at a protein concentration of 0 25 mg/ml eluted as whole subunits (13) from a Sephadex G-200 column equilibrated with borate buffer when reassociated at a concentration of 0 04 mg/ml the main peak eluted with unchanged position but a second peak eluted with the position of half subunits (13) No free light chains were observed when the products were filtered through Sephadex G 200 equilibrated with 0 1 N propionic acid made 6 M in urea

These observations indicate that when IgM is reduced at low protein concentrations covalently linked half subunits are also formed whereas only covalently linked whole subunits are formed at high protein concentrations This is most easily explained by assuming that a state of rapid equilibrium exists between whole and half mercaptoethanol reduced subunits (2 12, 13), and that formation of mixed disulphides can only take place with SH groups not exposed to the corresponding group on the other chain The extensive formation of mixed disulphides with SH groups involved in the intersubunit disulphide bonds indicates that the equilibrium constant for the reaction $n(\mu_2L_2) \rightleftarrows (\mu_2L_2)_n$ is low

TABLE 1 *Influence of Dialysis on the Extent of Reassociation*

Changes of buffer: Oxygenfree	Changes of buffer: Equilibrated with atmospheric oxygen	Fast sedimenting material Per cent of total
0	1	20
0	2	55
[illegible]	3	68
0	4	67
0	5	68
1	1	49
2	1	68
3	1	[illegible]
4	1	82

Reassociation procedure 2 To investigate whether reduction in the amount of ME by dialysis against several changes of buffer equilibrated with atmospheric oxygen increases the yield of reassociated material, [illegible] mg/ml portions of IgM were dialysed against 1–5 changes of buffer As shown in Table 1, the yield of fast sedimenting material increased from 20 to [illegible] per cent

Reassociation procedure 3 The concentration of ME was reduced by dialysis against 1 to 4 changes of degassed (oxygen free) buffer and the disulphide bridges subsequently reestablished by dialysis against buffer equilibrated with the atmosphere The results (Table 1) showed a marked increase in yield of fast sedimenting material, more than 80 per cent reassociated when the reduced protein was dialysed against 3 or 4 changes of degassed buffer prior to oxidation Alkylation prior to the

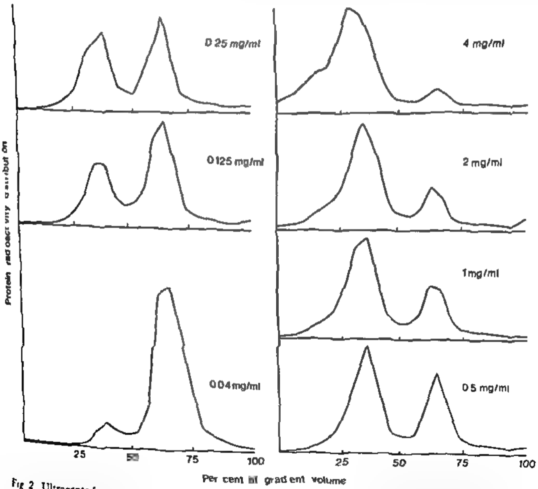

Fig 2 Ultracentrifugation patterns in 10–40 per cent sucrose gradients of mercaptoethanol reduced and reassociated IgM at different concentrations The top of the density gradients are to the right The ordinate indicates the relative protein concentration in the various fractions as determined by distribution of radioactivity

reformation of the disulphide bonds completely inhibited the formation of fast sedimenting material

The increased yield of fast sedimenting material in procedure ■ indicates that some process, probably the formation of mixed disulphides, interferes with reassociation to fast sedimenting material in the other procedures

The reduced yield of fast sedimenting material (40 per cent) after reduction of 8 S subunits obtained by procedure 1 may be attributed to the formation of mixed disulphides which are not readily reduced with 0 2 M ME in physiological saline (9) or to the oxidation of the SH-groups to sulphinic or sulphonic acids The experiments with ^{14}C labelled iodoacetamide show that slightly more than one SH group per 8 S subunit was irreversibly modified under the applied reducing conditions, and the reassociation experiments indicate that the SH groups which give origin to the intersubunit disulphide bonds are the groups most easily modified

Reassociation at different protein concentrations The extent of reassociation was investigated at protein concentrations from 0 04 to 4 mg/ml To obtain maximal yield procedure 3 was used for reassociation The results (Fig 2) show that the amount of reassociated protein increased markedly with increasing protein concentration, they also indicate a change in the sedimentation pattern of the reassociated fraction with formation of material sedimenting faster than 17 S at protein concentrations over 1 mg/ml The sedimentation pattern of the fast peaks did not change when the concentration of the material applied on the gradient was varied

The results demonstrate the importance of working with sufficiently high protein concentrations The change in sedimentation pattern of IgM reassociated at protein concentrations above 1 mg/ml is interesting since it shows that also material sedimenting faster than 17 S can be formed to appreciable degrees

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A SENSITIVE MODIFICATION OF THE OUCHTERLONY TECHNIQUE DETECTION OF HEPATITIS ASSOCIATED ANTIGEN BY IMMUNODIFFUSION IN A CLOSED HEXAGONAL SYSTEM

T Traavik, J C Siebke and Elisabet Kjeldsberg

Several modifications of the Ouchterlony double diffusion system are routinely used in testing for serum hepatitis. Simplicity specificity and simultaneous detection of Au/SH antigen and antibody are the main advantages of these procedures while comparatively low sensitivity and delay in reaching the final result are the most serious objections. In the conventional microtechnique, the use of short inter well distances allows a fairly sensitive, and rapid development of precipitation lines (1), but may easily lead to blurring of the precipitation lines or to antigen/antibody imbalance The same objections are applicable to the more sensitive, but also more tedious matrix gel diffusion technique (2) Increased sensitivity has been observed when using larger wells (3) connected with a correspondingly larger consumption of reagents

Generally, most of the components applied in the peripheral wells in such open Ouchterlony systems diffuse in a direction away from the antibody-containing central well Immunodiffusion in a closed hexagonal system should lead to increased sensitivity, since the diffusion from the peripheral wells in such a system, is restricted to a sector one third of that in an open system A procedure based on this principle has been developed in this laboratory and has been termed closed hexagon immunodiffusion (CHI)

Hexagonal diffusion chambers (Fig 1) were made as follows Hexagonal perforations were made in discs of 3 mm Perspex preferentially of dark colour The sides of the hexagons were 1 cm, with the vertices curved to fit a 3 mm diameter gel puncher The perforated discs were welded on to a sheet of 3 mm transparant perspex using chloroform or alternatively glued to microscope slides with a silicone adhesive (Silastic 732 RTV) In the experiments, 0 45 ml of 1 1 per cent agarose (L'Industrie biologique Francaise) in PBS containing 0 02 per cent of sodium azide, was filled into the chamber, giving an average gel thickness of 1,7 mm Due to wall effects, the gel thickness was greater at the periphery of the hexagon After allowing the gel to harden in the cold for about one hour, 3 mm wells were cut in the vertices, and the centre of the hexagon Only freshly prepared gels should be used in order to avoid gel shrinkage which may lead to false deviations of the precipitation lines, due to chanelling along the walls of the chamber Furthermore to seal the wells, soft liquid agar was filled into them and rapidly removed with a Pasteur pipette, shortly before applying the sera

A comparison was made between the sensitivity of Au/SH antigen detection by diffusion in CHI and in normal Ouchterlony diffusion, using the same gel thicknesses, well diameters and inter distances Different dilutions of an Au/SH antiserum were tested against a series of twofold dilutions of an Au/SH antigencontaining serum High titre reference Au/SH antigen was applied in two diametrically opposite wells to take advantage of the reinforcement phenomenon After filling the wells the chambers were left for 24 hours in a moist atmosphere at room temperature and then for several days in the cold room Precipitation lines could normally be seen after 24 hours and were usually optimally visible after two days For all dilutions of antiserum including the optimal one the sensitivity of antigen detection was considerably higher in CHI than in conventional open immunodiffusion normally by a factor of 8 and occasionally by a factor of 16 When testing another antigen/antibody system in the same manner normal human serum versus goat antihuman IgG serum similar results were also obtained The difference in sensitivity is greater than would be expected from the threfold increase in sensitivity in concentration obtained in a closed hexagon This may be partly due to the uneven gel thicknes in the closed hexagon

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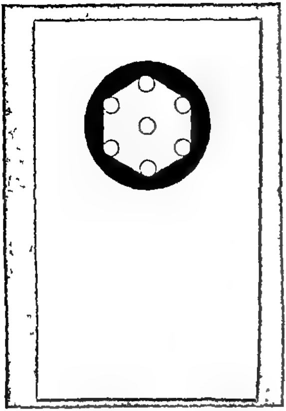

Fig 1 Outline of a closed hexagon immunodiffusion chamber showing the well pattern inside a perforated Perspex disc 2,25 × magnification Details described in text

The sensitivity of CHI was compared with that of counterelectrophoresis, using the commercial Hepa Screen system (Spectra Biologicals) The sensitivity for detection of Au/SH antigen was found to be 2 to 4 times higher in CHI During a current investigation of sera from presumptively normal African subjects, 20 Au/SH positive sera were detected by CHI, representing a frequency of about 10 per cent Three of these positive sera gave no lines in counterelectrophoresis, and some others only weak and inconclusive lines

In a reinvestigation of sera from patients suffering from various hepatic disorders, which had previously been tested in the diagnostic laboratory using a conventional 3 mm well diameter/3 mm inter well distance gel diffusion system 2 antibody and 1 antigen positive sera were detected in addition to 2 antibody, and 4 antigen, positive sera found in the conventional system

A beneficial effect of polyethylene glycol on immunodiffusion results has been reported previously (4) In the CHI system, sharper lines and frequently, a twofold increase in sensitivity were observed when applying 8 per cent polyethylene glycol 6000 into the wells 2–3 hours after application of the sera

The CHI system represents a suitable method for the detection of Au/SH antigen, or antibody in human sera Like the normal Ouchterlony diffusion, it is simple and safe in use, but considerably more sensitive CHI seems to be more sensitive than counterelectrophoresis, and has the advantage of giving the identity reaction

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TEMPERATURE OPTIMUM AND RABBIT BLOOD CELL CULTURE

K Thestrup Pedersen

The course of a lymphocyte transformation test (LTT) is influenced by several factors Investigations have shown the importance of cell density, presence or absence of adherent cells, concentration of antigen or of phytohaemagglutinin (PHA), duration of culture, pH and metal-cation requirements (1, 3, 4 5, 7)

LTT is traditionally carried out at 37 0 °C It appears that the normal rectal temperature of the rabbit is 39 6° C (6) The purpose of the present study was to investigate whether cultures of rabbit leucocytes would improve if carried out at physiological temperature

Materials and Methods

An amount of 30 ml of blood was drawn from the peripheral ear vein of a rabbit into a glass containing 600 I U of phenol free heparin The blood was taken from rabbits of which a few had previously been immunized with BCG vaccine Separation of red and white blood cells was done on an isopaque-methyl-cellulose mixture (2) The plasma containing leucocytes was centrifuged at 180 G (1000 rpm) for 10 minutes, washed twice in Hanks BSS and suspended in TC 199 culture medium containing leucocytes was centrifuged at 180 G normal rabbit serum Penicillin and streptomycin were added to concentrations of 100 I U /ml and 100 µg/ml respectively

Two identical sets of cultures were incubated in a water bath in 37 0° C and 39 0° C, respectively PHA (Wellcome) was dissolved in 5 0 ml of sterile saline and added to the PHA-containing cultures usually in a standard dose of 0 010 ml PHA/ml medium There was no addition to control cultures Triple cultures were normally made depending on the number of lymphocytes available Every culture contained 1 × 10⁶ lymphocytes per ml medium

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The total volume of the culture was 2 0 ml The cultures were flushed with atmospheric air containing 5 per cent carbon dioxide, and then incubated in water bath at 37 0° C or at 39 0° C

After 48 hours 1 0 ml of medium was exchanged with fresh medium, and 0 2 microCi of thymidine-C-14 was added (specific activity 57 Ci/mol The Radiochemical Centre, Amersham) At 72 hours, 1 0 ml of the cell culture was harvested on glass-fibre filters (3) The filters were dried and incubated at 37° C with 1 0 ml of 1 per cent hyamine solutions for 24 hours Then 10 ml of Bray's scintillation liquid was added, and the vials were counted in a TRI CARB Liquid Scintillation Counter The results were expressed as mean counts per minute (CPM) after correction for quenching and efficiency The background activity was not subtracted

Results and Discussion

The results are shown in Table 1 In order to facilitate comparison of the results from the individual rabbits the index (I) between the results from cultures at 39 0° C and those from cultures at 37 0° C was calculated

It appears from the table that all indices are greater than 1 00 This means that there was a higher rate of DNA synthesis in the cultures at 39 0° C than in those at 37 0°C The lowest value of I is 1 15, and the highest 3 13 This indicates an increase in the DNA synthesis between 15 per cent and 213 per cent when the temperature was increased

In two cases (rabbits Nos 2 and 6), three different concentrations of PHA were used simultaneously This is shown in Figure 1

The relatively wide variations in CPM and index may be explained partly by the use of different stock solutions of TC 199, PHA and serum, and partly by biological variations in the rabbits, including different optimum concentrations of PHA A concentration of PHA of 0 005 and 0 0025 ml PHA/ml medium respectively, resulted in intense DNA synthesis in the cultures in 39 0° C, but not in those at 37 0° C This may be due to the less favourable growth conditions at 37 0° C

TABLE 1 *PHA Stimulation of Rabbit Blood Cell Cultures at 37 0° C and 39 0° C*

Rabbit No	Conc of PHA in ml per ml of medium	PHA-stimulated cultures CPM at 37 0° C	PHA-stimulated cultures CPM at 39 0° C	Controls 37 0° C	Controls 39 0° C	Index CPM at 39 0° C / CPM at 37 0° C
1	0 010	620	1848	479	83	2 98
2	0 005	7528	19828			2 63
	0 010	15730	23043			1 46
	0 015	16571	22611	–	2402	1 36
2	0 010	3122	4832	–	222	1 55
3	0 010	1467	2827	63	61	1 93
4	0 010	8673	11504	67	51	1 33
5	0 015	4600	6683	–	38	1 45
6	0 010	8636	18061	–	93	2 09
6	0 010	6378	13754	1147	2028	2 15
6	0 0025	10150	19902			1 96
	0 010	12753	20122	3294	3233	1 58
	0 015	15989	18316			1 15
7	0 010	14889	21133	4106	4562	1 42
9	0 010	6969	21815	–	99	3 13
211	0 010	7595	9625	630	3623	1 27
214	0 010	47574	60673	2766	1942	1 28

– not done due to shortage of lymphocytes
Background = 18–28 CPM

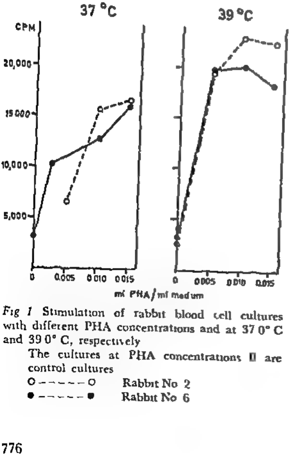

Fig 1 Stimulation of rabbit blood cell cultures with different PHA concentrations and at 37 0° C and 39 0° C, respectively

The cultures at PHA concentrations 0 are control cultures

o – – – – – o Rabbit No 2
• – – – – – • Rabbit No 6

Conclusion

The results of the study clearly show that stimulation of rabbit blood cells with PHA in the concentrations used yields a higher DNA synthesis at their physiological temperature (39 0° C) than at 37 0° C

The investigation shows how important it is to adapt the experimental conditions to the physiological conditions in the animal which is to donate cells for tissue cultures

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IMMUNOLOGICAL STUDIES OF THE L-PHASE VARIANTS OF SOME *E. COLI* STRAINS

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As a basis for the studies of the role of *Escherichia coli* L phase variants in urinary tract infections the immunological characteristics of the L phase variants of three *E coli* strains were studied (*E coli* O8 K8 H4, *E coli* O2 K1 H4 and *E coli* O15 K? H?) The L phase variants were induced by penicillin on solid media and could be subcultivated at least thirty times on penicillin free media without reverting to bacteria Antisera to the bacteria and L-phase variants were produced in rabbits Most of the antigens in the bacteria were also found in the L phase variants All L phase variants contained O antigen In the L-phase variant of *E coli* O8 K8 H4 K antigen was also found In all the micro-organisms an antigen with high electrophoretic mobility common to many *E colis*, Proteus and Pseudonomas strains was found There was enough O antigen in all the L phase variants to induce an O antibody response in rabbits similar to the O antibody response induced by complete bacteria

Some patients with typical clinical signs of pyelonephritis have no demonstrable bacteria in the urine—even on multiple testing Some of these patients may have an infection caused by a virus or they may have a focal bacterial pyelonephritis with no transfer of bacteria to the urine Many reports, however, have proposed L phase variants of bacteria which do not grow on regular media, to be an important aetiological factor (2, 4–7, 9, 10 17) The fact that urine is often slightly acid with a high content of electrolytes, combined with the hyperosmotic milieu of parts of the kidney, favours the survival of L-phase variants As they are less sensitive to some antibiotics than the corresponding bacteria, they can also survive a period of antibiotic treatment *Domingue et al* and *Gnarpe* have, on the basis of their own investigations and studies of the literature, estimated the frequency of isolation of L-phase variants in the urine of patients with urinary tract infection and negative bacterial cultures at around 5 per cent (5, 7)

As a basis for studies of the role of *E coli* L-phase variants in urinary tract infections the aim of this study was to investigate the immunological characteristics of the L phase variants of some *E coli* strains as compared to the original bacteria

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MATERIALS AND METHODS

Bacteria of the *E coli* strains G 3404/41 (Sero type O8 K8 H4) U9/41 (Serotype O2 K1 H4)

and F 7902/41 (Serotype O15 K? H?) from the WHO International Escherichia Centre, Statens Seruminstitut, Copenhagen, were used For morphological studies the bacteria were cultivated on blood agar plates made with placental agar containing 5 per cent defibrinated horse blood and on Drigalski agar made according to the description by *Kauffmann* (18), however, nutrient agar was used instead of placental broth and Danish agar The L phase variants of the same strains were cultivated on a solid, special medium This medium was based on the TY-1 medium for cultures of *E coli* in shaking flasks (11) To the medium was added per 1,000 ml sucrose 100 g, horse serum (inactivated 56°, 30 minutes) 100 ml and Agar Noble (Difco) 10 g pH was adjusted to 7 1 Plates were made both with and without penicillin G (1,000 IU/ml) The penicillin free medium was also used in liquid form in which agar was omitted

L phase variants were induced on the penicillin containing plates These L phase variants were repeatedly subcultivated as follows Small agar pieces containing some colonies of L phase variants were cut in the agar plates These pieces were transferred to 5 ml of the liquid special medium and shaken for five minutes After this, the liquid medium was poured on the plates for growth of L-phase variants (about 1 ml/plate) After about four subcultures, penicillin free plates could be used

The osmotically stabilized, solid medium showed surface growth of microorganisms that fulfilled the following criteria of L phase variants (22) Growth with the type of colonies, 'L colonies', first described by *Klieneberger Nobel* (19) in contrast to ordinary bacterial growth on solid media No growth of L-phase variants was obtained on the media commonly used for cultivation of *E coli* Ethanol fixed, gram stained smears from the colonies contained rounded, gram negative, poorly stained, spherical or pleomorphic bodies, but no rods In the experiments, both L phase variants from penicillin containing media and from penicillin free media were used The latter could be subcultivated on the penicillin free medium at least thirty times without reverting to the original bacteria

Antigen preparations of acetone dried bacteria and L phase variants were made by veronal buffer extraction, VE antigen (12) Sterility of these VE antigens was tested by cultivation both on ordinary media and on the special medium for L phase variants The antigens from the L phase variants were also microscopically examined to exclude a presence of dead bacterial cells

Purified O8 and O2 *lipopolysaccharide* (O8 Lps, O2 Lps) prepared according to *Westphal et al* (30) was kindly supplied by Drs *B* and *K Jann* at the Max Plank Institute fur Immunobiologie, Freiburg In the immunodiffusion studies they were used at a concentration of 1 mg/ml The O15 antigen was prepared by autoclaving the VE antigen from the O15 *E coli* strain at 120° C for 30 minutes

Antisera to the whole bacteria, OKH antisera, and to the O antigens, O antisera, were produced as earlier described by *Holmgren* (12) Antisera to L-phase variants were produced correspondingly to OKH antisera, using the harvest from the plates after at least four subcultures which were washed in 5 per cent saline and killed with formalin (0 5 per cent) The effectivity of the killing with formalin was checked each time The numbers of L phase variants per ml were counted in a Buchner chamber in order to arrive at corresponding numbers of bacteria injected Before each injection, the material was cultivated and microscopically examined to exclude bacterial contamination Blood samples from each animal were tested for sterility after each injection of L phase variants

For hyperimmunization, two rabbits were given eight injections at 5 to 8 day intervals The following volumes of micro-organisms (containing 10^9 micro organisms/ml) were administered 0 25 ml 0 25 ml, 0 50 ml, 0 50 ml, 1 0 ml, 1 0 ml, 2 0 ml, 2 0 ml In the first four injections, formalin killed bacteria were used Two rabbits were used for each of the following micro-organisms (except for *E coli* O8 K8 H4 L phase variants where we used five rabbits) *E coli* O8 K8 H4 bacteria, *E coli* O2 K1 H4 bacteria, *E coli* O2 K1 H4 L-phase variants from penicillin containing media, *E coli* O2 K1 H4 from penicillin free media, *E coli* O15 L phase variants from penicillin containing media and *E coli* O15 L phase variants from penicillin free media. Two rabbits were given a single injection of 0.5×10^9 *E coli* O8 K8 H4 followed by the same booster dose about two months later The L-phase variants of this strain was also administered to three rabbits, following the same immunization schedule and given in the same amount Still another two rabbits were given the above mentioned amount of bacteria and, about two months later, the same number of L phase variants as a booster dose Antiserum to common protein antigen, cpa was made by hyperimmunizing rabbits with this purified antigen (13)

Indirect haemagglutination, IHA, of serum samples was performed as earlier described (15) using the supernatant of boiled centrifuged bacteria as antigen To each haemagglutination test, hyperimmune sera of known titres were added as controls. Reduction of serum samples with beta mercaptoethanol was also done as described in this paper (15) *Absorption of O antibodies in the* antisera to the L phase variants of *E coli* O8 K8 H4 and O2 K1 H4 was done by adding O8 Lps and O2 Lps respectively, to a final concentration

of 2.5 mg/ml and incubation at 37°C for 45 minutes and at +4° C overnight followed by centrifugation If necessary, this procedure was repeated several times Absorption of the antibodies to O15 antigen was done as mentioned above using VE antigen which had been autoclaved for 30 minutes at 120° C Absorption of O antibodies in the antisera to the bacteria was also done by mixing the same volume of antiserum and washed, packed L phase variants The mixture was incubated for 45 minutes at 37°C and kept overnight at 4° C Thereafter it was centrifuged at 4,000 rpm for 15 minutes The antibody activity was measured before and after absorption, using the indirect haemagglutination technique

Immunodiffusion analyses were performed by the microplate double diffusion in gel technique described by *Wadsworth* (29) O-antigen was quantitated by the *single radial immunodiffusion* method described by *Mancini et al* (20) *Immunofluorescence studies* of ethanol-fixed L-phase variants were made by the indirect technique using the O antisera produced with boiled bacteria and fluorescein labelled anti rabbit immunoglobulin As negative controls served normal rabbit serum with no antibodies to the used three *E coli* strains detectable by indirect haemagglutination and O antisera to other *E coli* strains (U5/41, serotype O1 K1 H7, U4/41, serotype 04 K3 H5, Bi 7458/41, serotype O6 K2a, 2c H1, F 10018/41, serotype O18 K76 H14, E 14 a, serotype O22 K13 H1 and E 3 b, serotype O75 K? H5, all from the WHO International Escherichia Centre, Copenhagen, Denmark) Fluorescein conjugated anti rabbit immunoglobulin serum from sheep was supplied by the State Bacteriological Laboratory, Stockholm It contained 10 mg protein/ml and had a molar F/P ratio of 2 5 This antiserum was used in a dilution of 1/10. It gave no fluorescence of bacteria or L-phase variants when used directly on the smears A Leitz orthoplan microscope equipped for incident illumination, and with a high pressure mercury vapour lamp (Osram HBO 200) was used Primary filters were BG 38, BG 12 and KP 490 and secondary K 510 The photos were taken with Kodak Tri X and/or Ektachrome high speed

RESULTS

Identification and quantitation of O antigen in bacteria and L-phase variants In the double diffusion and immuno electrophoretic analyses we used four *E coli* O8 K8 H4, four *E coli* O2 K1 H4 and two *E coli* O15

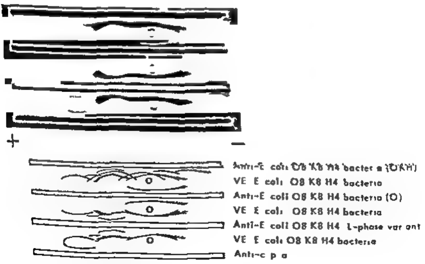

Fig 1 Immuno-electrophoretic pattern (5V/cm, 70 minutes) using antiserum to living (OKH) and boiled (O) *E coli* O8 K8 H4 bacteria and their corresponding L phase variants Antiserum to the common protein, c p a, was also used In the round basins Veronalbuffer (VE) antigen from the bacteria

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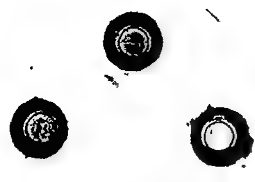


Fig 2 Double diffusion analysis In the upper basin, purified O8 lipopolysaccharide Lower left anti *E coli* O8 K8 H4 bacteria Lower right anti *E coli* O8 K8 H4 L phase variant

bacterial antigen preparations and four, three and two, respectively, of the homologous antigen preparations from the L-phase variants Using the corresponding antisera it was possible to show from repeated runs with different dilutions of antigens and antisera that the L-phase variants contained many of the antigens found in the bacteria (Fig 1) Antibodies in all of the six different *E coli* O8 K8 H4 bacterial antisera gave a coalescing precipitate with all of the corresponding ten different antisera to the L phase variant when tested against purified O8 lipopolysaccharide (Fig 2) Similar results were found with the two different *E coli* O2 K1 H4 bacterial antisera and the four antisera to the O2 K1 H4 L-phase variant if tested against O2 lipopolysaccharide, as well as with the four antisera to the 015 L phase variant if tested against autoclaved O15 VE antigen

Furthermore in the repeated immuno-electrophoretic runs it was possible to show the typical precipitate corresponding to O antigen antibody (12, 24), with all the above mentioned L-phase variant antigen preparations and antisera to the three L-phase variants

Indirect haemagglutination as well as the double diffusion experiments showed that the O8 and 02 antibodies in the antisera to the L phase variants could be absorbed by the corresponding purified O lipopolysaccharide preparation By repeated absorption, the O antibody titres measured by indirect haemagglutination could be reduced from about 16 000 to about 4 with 08 Lps The O2 titre decreased from about 8 000 to four after absorption with O2 Lps The O15 titre decreased from about 16 000 to about 16 using autoclaved 015 VE antigen If tested by double diffusion in gel the O precipitate

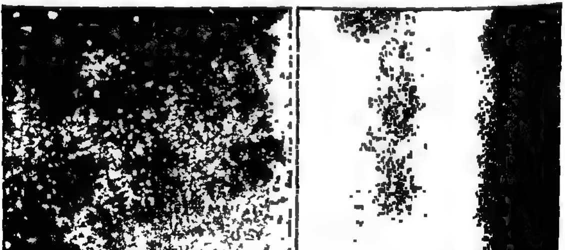


Fig 3 Immunofluorescence studies of the *E coli* O8 K8 H4 L phase variant using indirect technique with rabbit anti *E coli* O8 and fluorescein labelled anti rabbit immunoglobulin To the right negative control with normal rabbit serum

could no longer be detected for O8, O2 or O15 using absorbed antisera to L-phase variants.

The O antibodies could also be eliminated from the bacterial antisera by absorption with the corresponding L phase variants. O8 IHA titres of 32 000 could be lowered to about 4 by L phase variants from *E. coli* O8 K8 H4. O2 K1 H4 L-phase variants reduced O2 IHA titres from 16 000 to about 32.

The immunofluorescence studies of the L-phase variants were positive with all the corresponding bacterial O antisera (Fig 3). Tests with antisera against the six tested heterologous strains were negative, and the same applied to normal rabbit serum.

Repeated tests in single radial immunodiffusion plates using four different VE antigen preparations of *E. coli* O8 K8 H4 bacteria and four different VE antigens from the corresponding L-phase variants were done. As judged from comparison with dilution series of the purified O8 LPS, the amount of O antigen in the O8 K8 H4 L phase variants was about one fifth of that in whole bacteria of the same dry weight. The corresponding calculations of O2 antigen from the *E. coli* O2 K1 H4 bacteria and L phase variants were done with four bacterial VE antigens and three VE antigens from the L-phase variants. From dilution series of purified O2 Lps, the amount of O2 antigen in the L-phase variants was calculated to about one eighth of the same dry weight of bacteria.

Identification of antigens other than O, common to bacteria and L phase variants. By immuno-electrophoretic and double diffusion studies of the VE antigens from the three *E. coli* strains and the corresponding L-phase variants it was possible to identify some other antigens besides the O antigen. Repeated runs with different dilutions of the four *E. coli* O8 K8 H4 bacterial antigens and the four from the L-phase variants showed at least nine different antigens in the bacteria and at least four to five in the L phase variants (Fig 1). One antigen was found in all bacterial VE antigens as well as in the VE

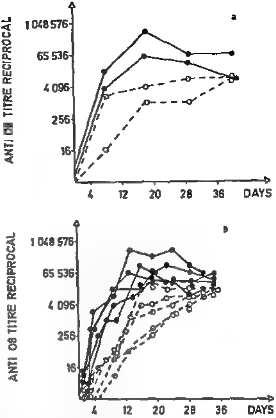
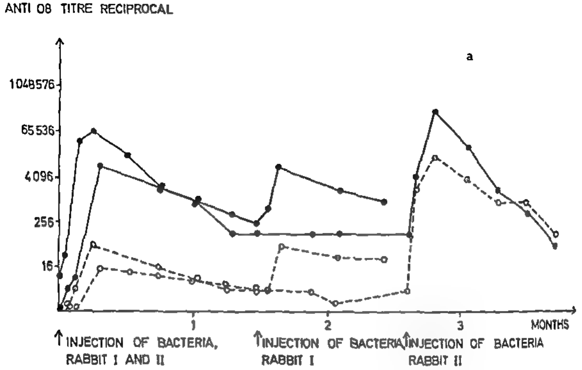


Fig 4. Hyperimmunization of rabbits with a) *E. coli* O8 K8 H4 bacteria and b) the corresponding L phase variants. ——— denotes antibody titre measured by indirect haemagglutination before and – – – after reduction with beta mercaptoethanol. Days after the first injection are indicated.

antigens from the L phase variants if tested against any of the six different bacterial OKH antisera or ten different antisera to the L phase variants. This antigen formed a very dense precipitate as is common to polysaccharide antigens. It had the electrophoretic mobility proposed for K8 (Fig 1). It was not immunogenic in rabbits if boiled microorganisms were injected.

It was possible also with the four *E. coli* O2 K1 H4 bacterial VE antigens to show eight to nine different antigens as compared to four to five in the L phase variants.

In the L-phase variants of *E. coli* O15, four to five different precipitating antigens including O15 were found using any of the four antisera to the L phase variants.



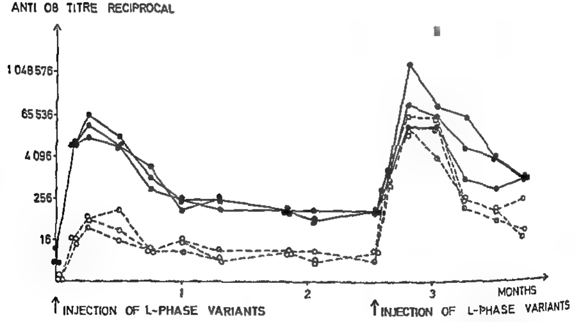


Fig 5 Immunization of rabbits with a) two different injections of *E coli* O8 K8 H4 bacteria b) two different injections of *E coli* O8 K8 H4 L-phase variants and c) priming with bacteria *followed* by a booster dose of L phase variants ——— denotes antibody titre measured by indirect haemagglutination before and – – – – after reduction with beta mercapto ethanol Days after the first *injection* are indicated

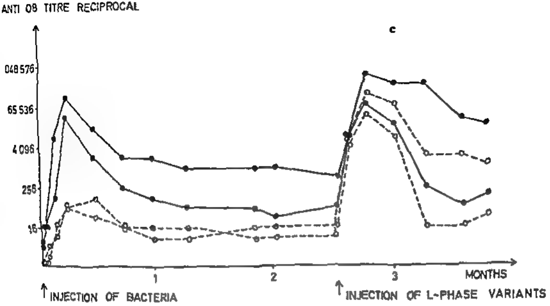
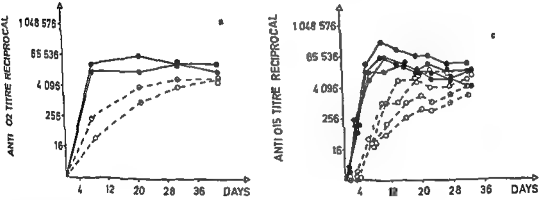


Fig 5



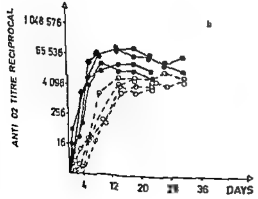


Fig [illegible] Hyperimmunization of rabbits with a) *E coli* O2 K1 H4 bacteria b) the corresponding L-phase variants and c) *E coli* O15 L phase variants ——— denotes antibody titre measured by indirect haemagglutination before and – – – – – after reduction with beta mercapto ethanol Days after the first injection are indicated

In all of the mentioned bacteria and L-phase variants we found an antigen with a high electrophoretic mobility. This could be identified as a protein, c p a, common to many *E coli* as well as to some Proteus and Pseudomonas strains (13)

The course of the antibody response to O antigen of bacteria and the corresponding L-phase variants The antibody response in two rabbits hyperimmunized with *E coli* O8 K8 H4 bacteria (Fig 4a) and in five animals hyperimmunized with the corresponding L-phase variants (Fig 4b) was studied by IHA. There was a marked O8 antibody response within a few days in all rabbits. Antibodies sensitive to as well as resistent to reduction with beta mercapto ethanol were found, the latter dominating the later part of the immunization schedule. No apparent difference was noted in the antibody response to bacteria as compared to L phase variants

Two rabbits given a single dose of 0.5×10^{8} *E coli* O8 K8 H4 bacteria (Fig 5a) and three immunized with the same amount of L phase variants (Fig 5b) showed very similar primary responses against O8 antigen. Besides, there was no apparent difference in the antibody response to a booster dose of *E coli* O8 K8 H4 compared to a booster dose of the corresponding L-phase variants (Fig 5a and b). In two rabbits primed with bacteria and boostered with L-phase variants a similar response was registered (Fig 5c)

The antibody response to *E coli* O2 K1 H4 bacteria as well as to the corresponding L phase variants was studied in six other rabbits (Fig 6). Two rabbits were each hyperimmunized with bacteria (Fig 6a), L-phase variants from penicillin containing plates and L phase variants from plates without penicillin (Fig 6b). In addition, two rabbits were immunized with *E coli* O15 L phase variants from penicillin containing plates and two with *E coli* O15 L-phase variants from penicillin free plates (Fig 6c). The O antibody response against these bacteria and L phase variants was similar to that found for *E coli* O8 K8 H4. About one month after the last injection into these eight rabbits immunized with L-phase variants they received a booster injection of 0.5×10^{8} L phase variants. There was a marked O antibody response with both beta-mercapto ethanol resistent and sensitive antibodies

DISCUSSION

Immunological studies of L phase variants from *E coli* are rather scarce. In a brief report from 1971, *Petersen et al* (25) showed that *E coli* L phase variants can evoke an antibody response if injected into rabbits, but they did not study this antibody response in detail. In a preliminary report *Kaijser et al* (14) showed that *E coli* L phase variants and the corresponding whole bacteria have many antigens in common and among these a quantity of O antigen sufficient to evoke an O antibody response similar to that of bacteria if injected into rabbits. In contrast, L-phase variants from Proteus have been extensively studied. *Minck et al* (23) showed that Proteus L phase variants can still have most of the antigens from the whole bacteria and, among these O antigen and H antigen that is flagella. *Vatuzis* reported studies of Salmonella and declared that it was possible to show long flagella even from the round spheroplasts (27)

In work with "cell wall defective microbial variants" it is essential that each author gives his definition of the micro organisms. This is necessary because of the lack of generally accepted definitions of the different wall defective phases of bacteria. In our studies we followed the definition of L phase variants presented by *McGee et al* (22) (See Materials and methods). The induction of the variants from the bacteria was done with penicillin. In the experiments with L phase variants from *E coli* O2 K1 H4 and O15 the tests were run in parallel with L phase variants growing on penicillin containing as well as on penicillin free media. Most of the experiments with L phase variants of *E coli* O8 K8 H4 were done with penicillin containing media. However L phase variants of

this strain proved to be stable for at least some thirty subcultivations even in the absence of penicillin. Both the L phase variants from penicillin free and those grown on the penicillin containing media showed the same immunological characteristics throughout this study.

Without relevant controls there is always a risk of bacterial contamination of the preparations of L-phase variants. To eliminate this risk, we always checked by cultivation and microscopic examination the VE antigens and preparations employed for immunization of the L-phase variants. Moreover, we hindered the variants from converting to bacteria after injection into rabbits, by injection of formalin killed L phase variants.

We found most of the bacterial antigens in the corresponding L-phase variants. There was sufficient O antigen in the L phase variants from all the three bacterial strains to induce an O antibody response in the rabbit. This antibody response was quite similar to the O antibody response evoked by homologous bacteria administered repeatedly or given as a single dose. The booster effect of L-phase variants was as good as that of bacteria after priming with bacteria of L phase variants.

Quantitation of O antigen by single radial immunodiffusion is somewhat unreliable if the molecular size of the antigens studied is unknown. We cannot state whether or not the O antigen in L phase variants differ from the O antigen in the bacteria. Thus the comparison of the amount of O antigen from bacteria and L phase variants could be misleading if transformation of bacteria to L phase variants also causes a physiochemical change of the O antigen. However, the fact that we could absorb the O8 and O2 antibodies in bacterial antisera with the corresponding L-phase variants indicates that they were at least antigenically similar.

The immuno electrophoretic analyses revealed a very dense precipitate with antiserum to the L phase variants of *E. coli* O8 K8 H4 against VE antigen from the bacteria or L phase variants. This antigen had the same electrophoretic mobility as the K antigen from the *E. coli* strain O8 K8 H4, as described by *Ørskov et al.* (24). It was also shown to precipitate only with antiserum against whole bacteria or L phase variants, not with antiserum to boiled bacteria. Thus, we assumed this antigen to be the K8 antigen.

We were not able to demonstrate in this way the K1 antigen in the *E. coli* O2 K1 H4 L phase variants. Furthermore, in recent unpublished studies we found no K1 antibodies as measured by indirect hemagglutination after injection in rabbits of these L-phase variants.

In all of the studied *E. coli* bacterial strains and L phase variants, we found an antigen, probably a protein, with high electrophoretic mobility. The role of this antigen or its localization in the cell is not definitely known. Its antibodies are not protective against infections (13).

Studies of experimental infection in animals have suggested that *E. coli* O as well as K antibodies may be protective (15, 16, 31). From the aforementioned it is evident that protection against infections caused by L-phase variants may also be possible. Thus the proposed tendency of L phase variants to persist in different tissues (8, 21, 26) may probably not be explained by the postulation that protection of O and K antibodies is escaped if these antigens are lost, always provided that L phase variants *in vivo* have the same immunological characteristics as those we have studied *in vitro*.

It is well known that pyelonephritis caused by *E. coli* evokes an antibody response (1, 3, 28). Our present results do not exclude the possibility of L phase variants without O or K antigens. However, in the L phase variants so far studied we could show both O and K antigen. Thus an infection of the renal parenchyma by *E. coli* L phase variants might also give an O and K antibody response. Furthermore remaining L phase variant persisting in the kidneys may possibly stimulate to increased levels of antibodies. This might be the explanation of long lasting increases in O antibody titres observed in a

few patients with a history of urinary tract infection but with no positive urinary cultures (1)

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EFFECT OF IRRADIATION ON ESTABLISHED DELAYED HYPERSENSITIVITY

Suppression of skin reactions, recovery and the effect of cell transfer

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When rats immunized with bovine serum albumin in complete adjuvant were irradiated 10 days later with 800 rads their ability to display a positive skin reaction to the test antigen was suppressed for 4–5 days Thereafter recovery was evident in spite of persisting low blood leucocyte levels Specific cells were demonstrated in the animals by migration inhibition of peritoneal cells two days after irradiation, when skin reactions were negative On the other hand transfer of normal bone marrow cells corrected the ability to mount skin reactions but spleen, thoracic duct or lymph node cells did not It seems that post irradiation suppression of skin reactions does not depend on damage of specific cells but depends on shortage of a bone marrow derived cell type participating in skin reactions as a nonspecific component

Irradiation before immunization strongly depresses antibody formation (13), and it also inhibits the development of delayed hypersensitivity (15, 16) Although irradiation after immunization has only a minor effect on antibody production (13), *Lennox et al* (6) found that rabbits displaying tuberculin hypersensitivity lost their ability to react to tuberculin skin tests for a short period of time after whole body irradiation Later, *Volkman and Collins* (19) reported similar results with mice using a footpad swelling test to detect delayed hypersensitivity By contrast it has been reported that a high dose of irradiation after sensitization with DNCB could not prevent the development of contact dermatitis in guinea pigs to a test inoculation with DNCB (8)

This investigation examines the effect of whole body irradiation on established delayed hypersensitivity against bovine serum albumin (BSA) in rats and the effect of postirradiation cell transfer on the immunological recovery of the animals

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MATERIALS AND METHODS

Inbred male Sprague Dawley rats were used They were kept in large well ventilated cages and fed with commercial rat pellets bread cheese and water

Sensitization

Animals were sensitized with bovine serum albumin (Armour Pharm Co Eastborne, England) emulsified in complete Freund's adjuvant ((Bayol F oil (Esso) 8 5 volumes Arlacel (Atlas Powder Co Wilmington, Delaware) 1 5 volumes and heat killed dried tubercle bacilli at a final concentra

tion 3 mg/ml) so that the final concentration of BSA was 5 mg/ml 0 1 ml of this material was injected into a hind footpad of each animal

Skin Tests

0 1 ml of BSA solution containing 300 μg BSA/ml was injected intradermally Skin reactions were examined 3 and 24 hours after injection Two perpendicular diameters of the reactions were measured, and the induration was assessed on an arbitrary scale from — to + + + + Each animal was tested not more than three times with a minimum of 72 hours between each injection

Skin Reaction Histology

Biopsies were taken from skin reaction sites at 24 hours, and paraffin sections cut from these were stained with Giemsa

Migration Inhibition Tests

Macrophage migration inhibition was tested according to *David et al* (3) Peritoneal exudate was induced with 2 5 per cent starch gel Cells were collected 3 days later with balanced Hanks salt solution containing heparin (1 1000), washed once, and suspended in Eagle's medium containing 100 iu penicillin/ml and 10 per cent inactivated normal rat serum Cell suspension was drawn into 50 μl capillary tubes and one end of capillary was sealed with clay After centrifugation capillaries were cut at the cell fluid interface and pairs of cell-filled tubes placed in chambers The chambers were filled with Eagle's medium containing 200 μg BSA/ml or without this antigen At least two chambers containing antigen and two control chambers were made from each After incubation for 24 hours the area of migration was drawn (magnification × 30) and measured by a planimeter The extent of migration was expressed as per cent migration with antigen compared to that without antigen Average values used in comparisons were means of at least four capillaries

Antibody Determinations

Animals were bled from the tail, and anti BSA antibodies were determined by passive haemagglutination of tanned sheep red blood cells coated with BSA (14) Sensitivity to mercaptoethanol was determined by adding 2 mercaptoethanol (Fluka AG Buchs SG Switzerland) to each tube of serum dilution giving a final concentration of 0 05 M Coated red blood cells were added after 30 minutes incubation at 37° C (11)

Cell Counts

Blood leucocytes were counted from tail blood and blood smears were prepared for differential counts using Giemsa stain

Cell Transfer

Cell suspensions were prepared from spleen, lymph nodes and bone marrow (femoral and tibial) of normal syngeneic rats The thoracic duct was cannulated for 12 hours to obtain duct lymphocytes The number of nucleated cells was counted and the percentage of viable cells was determined by trypan blue exclusion Cell suspensions in Hanks salt solution with heparin (1 1000) were injected into tail veins of recipient rats after irradiation

Irradiation

A Siemens gammatron-3 telecobalt unit was used for whole body irradiation of rats using previously described techniques (16) During different experiments the absorbed dose rate varied from 45 rad/min to 150 rad/min

RESULTS

Animals were immunized with bovine serum albumin (BSA) in complete adjuvant They were irradiated with 800 rads of whole body irradiation 10 days after immunization and then skin tested during the following 9 days Separate groups of rats were subjected to migration inhibition studies and cell transfer experiments

Skin Reactions

Separate groups of four rats were skin tested at 6 and 2 hours before irradiation and 2 6, 12, 16 and 24 hours after it When test injection was given from 6 hours before to 12 hours after irradiation animals had smaller reactions than nonirradiated controls but almost all had clearly positive reactions However in the last two groups of the first day only a few reactions remained weakly positive, most having reverted to negative After the first day, groups of 9 rats were skin tested at periodic intervals Most of the tests made 2–3 days after irradiation were negative By the 4th and 5th postirradiation days, some signs of recovery of reactivity could be seen and by the 7th and 9th days full recovery was obvious All animals tested 9 days after irradiation had clearly positive reactions During this recovery phase some animals had large visible reactions (16–17 mm), but induration of only + grade, (Fig 1)

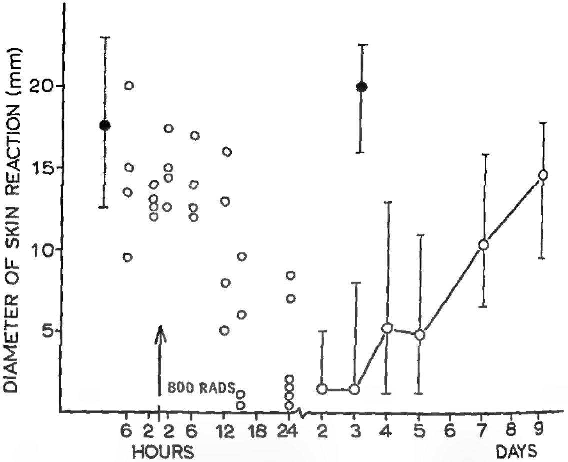

Fig 1 Skin reaction diameters in rats irradiated with 800 rads 10 days after immunization with BSA Separate open circles represent individual tests made during the irradiation day The curve represents mean values with ranges of 9 skin reaction diameters made later Means and ranges of 9 skin reactions in nonirradiated rats at the 10th and 13th day after immunization are included (solid circles) Each mark is on the day of injection

Separate groups of 5 and 6 rats were irradiated with lower doses (600 and 400 rads respectively) and tested 2 and 5 days after irradiation In the 600 rad group all reactions were negative at the 2nd day but clearly positive at the 5th day In the 400 rad group all reactions were positive though smaller than reactions in nonirradiated animals at the 2nd day test

To detect possible Arthus reactivity skin tests were examined 3 hours after injection The only positive three hour reactions during the study were one weak reaction in the non irradiated control group on the day of irradiation and in 5 of the 9 control rats 3 days later

Histology of Skin Reactions

Several biopsies of 24 hour skin reactions were examined microscopically Biopsies from 9 macroscopically positive reactions taken on the day after irradiation had a moderate degree of mixed cell infiltrate with polymorphonuclear cells predominating Three reactions from nonirradiated controls from this same day had histology typical for delayed hypersensitivity with heavy mainly perivascular mononuclear cell infiltrates Seven of the 9 biopsies of negative phase reactions taken 2 to 3 days after irradiation had slight mainly polymorphonuclear infiltrates without perivascular involvement These were indistinguishable from reactions in nonimmune ani

mals In the remaining two biopsies of this group there were slight mononuclear cell infiltrates The latest 12 biopsies taken from the 7th and 9th day after irradiation all showed varying degrees of mainly mononuclear cell infiltrates especially in perivascular spaces The cellularity of the infiltrate was highest on the 9th day when the reactions were also microscopically strongest

Antibodies

Anti BSA antibodies were measured from samples taken during a 15 day period after irradiation A significant drop in titres was seen (p values, when compared to nonirradiated controls at the 14th day < 0.01, and at the 17th and 20th day < 0.001), and the lowest values were detected 7 days after irradiation A week later the titres had considerably recovered, but were still lower than control titres ($p < 0.05$) (Fig 2) Treatment with mercaptoethanol had no effect on the titres

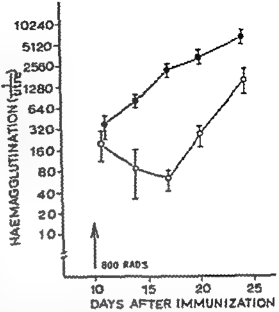

Fig 2 Mean anti BSA titres of 10 rats irradiated with 800 rads (open circles) and of 5 non irradiated rats (solid circles) Bars show ± one standard error

Blood Leucocyte Numbers

Blood polymorphonuclear, monocyte and lymphocyte values were recorded before irradiation and 2 hours, 1, 3, 7 and 10 days after it (Fig 3) After a small initial rise in monocyte and polymorphonuclear numbers detected 2 hours after irradiation there was a drastic reduction of all cell types within 24 hours A slight recovery of mononuclear cell numbers, which considering lymphocytes was significant when compared to the 3rd day value ($p < 0.05$) was seen at the 7th postirradiation day

Migration Inhibition Tests

Some of the rats were used for migration inhibition tests These animals were not subjected to skin tests Two days after irradiation peritoneal cells were collected and used for migration inhibition tests using 200 μg BSA/ml of culture fluid This caused a migration inhibition to 36 8 per cent migration in sensitized irradiated animals compared to 51 3 per cent in similar sensitized but nonirradiated rats This difference was not statistically significant (t test) Peritoneal exudate cells from nonimmune rats irradiated with 800 rads were not inhibited by BSA (Table 1)

Cell Transfer Experiment

A separate group of immunized rats were also irradiated with 800 rads Within the first 2 postirradiation hours these animals received normal syngeneic spleen lymph node thoracic duct or bone marrow cells Some of the bone marrow spleen and thoracic duct cell

TABLE 1 *Peritoneal Cell Migration Inhibition with BSA 2 Days after Irradiation with 800 Rads**

	No of rats	Migration %	SD
Sensitized nonirrad	7	51 3	16 8
Sensitized irrad	9	36 8	18 9
Normal irrad	6	98 5	11 7

* 10 days after immunization

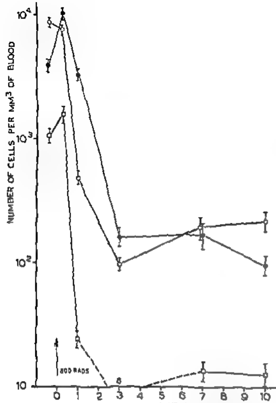

Fig 3 Blood lymphocyte (O), monocyte (□) and polymorphonuclear (●) counts in irradiated rats Marks represent mean value of leucocytes in 8 rats ± one standard error

recipients were skin tested with BSA 2 days after irradiation These animals had weak ± reactions Separate rats were tested 5 days after irradiation and by this time bone marrow recipients showed reactions of grade + + to + + + + Rats which received other cells were similar to those that received no cells and showed — to + + reactions except for two spleen cell recipients which showed + + + reactions (Table 2)

DISCUSSION

Rats were irradiated ten days after immunization with bovine serum albumin when *Arthus reactions were not expected, but* strong delayed reactions were already elicitable (16) Irradiation with 800 rads caused progressive suppression of skin reactions slight on the day of irradiation and maximal 2 days after *irradiation*, when most reactions were negative even in the group which received only 600 rads Some days later animals gradually *recovered their ability* to display skin reactions so that on the 7th post irradiation day recovery was clear A slight rise in blood mononuclear cell numbers was seen simultaneously with this recovery, but cell numbers were still depressed *Lennox et al* (6) reported a varying suppression of tuberculin reactions in rabbits with negative reactions in some animals given only 200 r and higher doses leading to suppression for several weeks In the present experiment 800 rads produced a clear suppression of reaction in the rat but duration was shorter than one week and recovery quite uniform The lower doses used had weaker effects and 400 rads did not convert reactions to negative The

TABLE 2 *Induration Grade and Si e of Skin Reactions 5 days after Irradiation*

Transferred cells	Without cell transfer	Bone marrow 5×10^8	Spleen 8×10^8	Thoracic duct 5×10^8	Lymph node 5×10^8
	—	+ + 16	± 11	± 11	± 11 5
	+ 6 5	+ + + 18	+ 11	+ 11 5	+ 11
	± 9 5	+ + + 19 5	+ 11	+ 12	+ 12 5
	± 11 5	+ + + 21 5	+ 13	+ 14 5	+ 14 5
	+ 13	+ + + 19	+ 13 5	+ + 16 5	+ + 14 5
	+ 15	+ + + + 20*	+ + + 19		
			+ + + 20		

* Mean skin reaction diameter of this group differs significantly from irradiated controls ($p < 0\ 001$ t test)

results of *Volkman and Collins* (19) with the footpad swelling test in mice showed a similar timetable of recovery

Skin reaction histology did not give information clearly different from that from macroscopic reactions The relatively high number of polymorphonuclear cells seen in biopsies taken on the day after irradiation could be due to the relatively high percentage of these cells in the circulation during the first postirradiation day

That skin reactions of Arthus' type were not found in irradiated rats in contrast to nonirradiated rats is at least partially explained by the fall in antibody titres This fall and later rise in antibody titres proceeded more slowly than did skin reactions The main reason for this phenomenon could be the loss of antibodies through the gut wall in irradiated animals (9) because both antibody producing cells (12) and the serum response (13) should be resistant to irradiation at this stage However, it is not probable that an effect of this size could be due only to non specific antibody loss Anti BSA antibody synthesis was probably impaired too

The cellular infiltrates of delayed hypersensitivity reactions are considered to be formed from a specific thymus derived cell population (22) and a nonspecific co reacting cell type mainly originating in bone marrow (4, 7) Most of these haematogenous cells (5) should vanish along with other circulating leucocytes during the first day after whole body irradiation *Pepys* (10) and later *Volkman and Collins* (19) suggested that the need for some circulating cell type could explain postirradiation nonreactivity in delayed hypersensitivity It is known that passive transfer of delayed hypersensitivity does not succeed in irradiated recipients (2) However passive transfer is not prevented when irradiated cells are transferred to normal recipients (1) This speaks in favor of suppression by irradiation of some nonspecific cell component in the recipients The present work adds further data to this Using an *in vitro* correlate of delayed hypersensitivity it is shown that rats still have enough functioning specific cells to cause inhibition of migration of peritoneal macrophages two days after irradiation, when skin reactions are negative The cell transfer experiment shows that bone marrow cells can effectively correct the defect in the ability to display skin reactions Lymph node and thoracic duct cells, known to be active in the inductive stage (17) had no effect Spleen seems to contain effective cells for both functions (17)

It seems that post-irradiation nonreactivity does not depend on loss of active immune cells but depends on shortage of bone marrow dependent co reacting cells (7) probably found in the circulation as monocytes (20), and in the skin reaction as macrophages Peritoneal macrophages, which in rats have their origin in the bone marrow (18), have recently been shown also to correct this postirradiation nonreactivity in guinea pigs (20) A striking feature is that for expression of delayed hypersensitivity in the recovery phase quite low blood mononuclear cell levels are adequate This could be a sign of a very effective cell capture mechanism in reaction areas or of active local cell proliferation

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THE PROTECTING CAPACITY OF NEUTRALIZING ANTIBODIES BY DISTEMPER VIRUS INFECTIONS IN MINK

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By challenge experiments on minks it has been shown that distemper vaccines may provoke formation of neutralizing antibodies in good titres without giving protection In small scale experiments it was found that mink kits even at the age of 4 weeks might be protected through vaccination, provided the mothers had not been vaccinated In kits born of vaccinated mothers, successful vaccinations may apparently be carried out by the age of 11 weeks, but if applied before this time, maternal antibodies seem to interfere with the vaccination At 4 weeks, maternal antibodies alone may give protection

In connection with vaccinations with killed virus vaccines against measles and respiratory syncytial virus infections in man it has been found that circulating antibodies did not protect against disease Such a vaccination may even result in a more severe disease if a later natural exposure to the virus actually occurs (e g *Fulginiti et al* (1967), *Norrby et al* (1966), *Kim et al* (1969) Consequently testing antigenicity cannot always be the proper or sufficient way of testing the efficiency of a killed virus vaccine The proper testing of certain vaccines for use in man may therefore present difficulties as long as more appropriate tests are not developed

Testing of veterinary vaccines may be carried out as true challenge experiments Experience gained in the use of dog distemper virus vaccine may therefore be useful for the understanding of measles vaccination, dog distemper virus and measles virus being very closely related

During a distemper epizootic in 1969 in the mink farms of Northern Jutland a number of minks were exposed to natural infection after vaccination with one of the two live attenuated vaccines employed (*Hansen* (1971)) Out of 9 unvaccinated controls introduced in the farm, 7 died from distemper All vaccinated animals had antibody titres of such levels that they would have been considered protected and one vaccine did provide full protection none out of 19 animals died or showed signs of disease Vaccination with the other vaccine proved to be without value as 17 out of 20 animals in this group died from distemper The work to be presented here was carried out as a further investigation into the problem of vaccine producing antibody formation but not protection The effect of maternal antibodies

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in connection with early vaccinations was studied also

MATERIAL AND METHODS

The experimental farm The experiments were carried out in a small farm set up for experimental use on Fejø, a small Danish island (*Hansen et al* (1973))

The animals were obtained from commercial breeders The farms had no previous contact with dog distemper virus unless specifically mentioned

The vaccine employed was Candur–S vaccine produced by Behringwerke from the Rockborn strain in dog kidney cell cultures In one experiment a sample from an egg vaccine produced by the Danish State Veterinary Serum Laboratory from the Ondestepoort strain was used also.

Virulent distemper virus Lyophilized samples of one batch of the Snyder Hill strain of dog distemper virus was kindly supplied by Behringwerke who suggested that the lyophilized material could be diluted 1/10 and still be infectious for dogs and ferrets Preliminary experiments (*Hansen et al* (1973)) had shown that the virus material could be diluted 1/50 and still kill 4 out of 5 minks using 1 ml doses subcutaneously Consequently this dilution was used throughout the experiment With increasing age of the experimental animals the resistance towards the virus as well as the incubation time were found to increase In spite of this the challenge dose was kept

TABLE 1 *The Antigenicity and Protective Capacity of Different Vaccine Samples*

	Vaccine	Antibody titre at challenge	Average	Mortality
I	Candur S, normal dose	1000		
		1000		
		1000		
		500		
		500	700	0/9 = 0%
		500		
		500		
		500		
		–*		
II	as I but heat treated as suspension for 2 hours at 56° C	250		
		250		
		200		
		200		
		100	130	4/10 = 40%
		50		
		50		
		50		
		50		
		–*		
III	A vaccine stored as suspension for 12 months at room temperature	200		
		200		
		100	120	2/6 = 33%
		100		
		50		
		50		
	Unvaccinated controls	10§	negative	3/9 = 33%
		10§		
		the remaining seven < 10		

* Serum not obtained or serum infected

§ Titres of 10 or less are considered unspecific (see Hansen et al (1972))

constant. The clinical diagnosis of distemper was confirmed histologically

Neutralization tests were carried out in dog kidney cell cultures as previously described (*Hansen et al* (1973)) The virus employed was the third passage of the Candur S vaccine strain The serum titres were expressed as the reciprocal of the serum dilution which represented 50 per cent endpoint of neutralization The sera were tested in 5 fold dilution steps starting with 1/10

EXPERIMENTS

1 *Vaccination with Thermally Inactivated Vaccines*

The following experiment was set up in order to test the hypothesis whether a live vaccine may become inactivated and thus be without protective capacity, but still retain antigen in a vaccine dose sufficient to produce antibodies

3 groups of dark mink of the same age and from the same breeder were vaccinated by subcutaneous injection of 1 ml One additional group served as unvaccinated controls

The following vaccines were used

I Dog kidney cell culture vaccine treated in prescribed manner ([illegible]

[illegible] pressed in 50 per cent protective capacity for mink and ferret was found to be 256–1024 (*Hansen et al* (1973))

II The vaccine of I suspended and treated for 9 hours at 56° C prior to use

III A sample of egg produced vaccine which had been kept as a suspension for 12 months at room temperature The sample was sufficient for 6 animals Originally this vaccine had a protective titre of 128 (*Hansen et al* (1973)) when challenge in mink and ferret was carried out

Four weeks after vaccination the animals were challenged with virulent virus The challenge dose used in this experiment caused death in 3 out of 9 unvaccinated controls The animals were bled immediately before the challenge inoculation 2 animals died before challenge for reasons unrelated to the vaccination

The antibody titres at challenge and the mortality of the minks after challenge are recorded in Table 1

The mortality in the two groups of animals vaccinated with vaccines treated in such a way (by heat treatment or prolonged storage as suspension) that active virus could not have survived corresponded with that in the control group Even so the vaccinations had caused antibody formation The neutralization test was carried out with 100 ID_{50} of test virus and the challenge dose proved to be low giving a mortality otherwise known to be quite normal in an epizootic of dog distemper in a mink farm

In the vaccination-control group I where the animals were given prescribed doses in the prescribed way, the antibody titres were higher, the average titre being about one 5 fold dilution step higher than that in the other groups, but all animals proved protected

TABLE 2 *Protection of 4 Weeks Old Kits by Vaccination of Mothers*

Animal groups	Mortality
Vaccinated females	0/5 = 0 %
Kits from these	1/13 = 8 %
Unvaccinated females	3/4 = 75 %
Kits from these	9/10 = 90 %

2 *Vaccination of Mink Kits*

Protection of 4 weeks old kits by vaccination of mothers In a small experiment using dark minks, the offspring of 5 females vaccinated 5 months before partus and 4 unvaccinated females were given challenge virus subcutaneously at the age of 4 weeks At the time of the challenge the vaccinated mothers had antibody titres of 1250 or more

The results of the challenge experiment is shown in Table 2

The challenge dose was high only one unvaccinated female and one of the kits in this group survived The females as well as their 4 weeks old offspring except one of the kits were protected by vaccination

Vaccination of young minks from mothers vaccinated during pregnancy From a farm which experienced a distemper epizootic the year before with a mortality among young animals of 38 per cent a number of pregnant pearl mink were purchased These breeders were vaccinated with aerosol spray vaccine (*Hansen* (1971)) about 4 weeks prior to partus The young ones were divided into 3 groups and the animals were vaccinated when they were 5 8 and 11 weeks old respectively The kits were given full prescribed vaccine dose subcutaneously A corresponding number of the kits were not vaccinated but kept as controls The kits were bled just prior to vaccination and the day before challenge The sera from the youngest were pooled before they were tested for neutralizing antibodies Challenge dose of virulent virus was given at the age of 5 months

As may be seen from Table 3 some protection of the kits was apparantly obtained in the animals vaccinated at 5 and 8 weeks, but when the kits were 11 weeks old they seemed to be fully protected by vaccination

TABLE 3 *Vaccination of Mink Kits from Vaccinated Mothers at Different Age*

Age of kits at vaccination	Average of antibody titre at vaccination	Average of antibody titre at challenge when the kits were 5 months old	Mortality
5 weeks	250	50	4/7 = 57 %
8 weeks	10*	250	5/7 = 71 %
11 weeks	negative(< 10)	≥1250	0/6 = 0 %
No vaccination of kits		negative(< 10)	14/16 = 83 %

* Not necessarily a specific titre

A Comparison between Vaccination of Mink Kits from Vaccinated Mothers and Kits from Unvaccinated Mothers

Pregnant minks were purchased from two commercial breeders The minks from one farm were vaccinated by injection 5 months before partus *Both sets of animals were divided into 4 groups* and vaccinated, using full dose of vaccine by injection Nine animals born of unvaccinated mothers served as unvaccinated controls

All the kits were given challenge at the age of 15 weeks The results of the experiment is compiled in Table 4

At the age of 4 weeks, kits of vaccinated mothers seem to be protected from fatal disease if they are vaccinated When this result is compared with those recorded in Table 2, it seems, however, as if these kits might have been better off without vaccination The results recorded in Table 4 regarding the offspring of unvaccinated mothers, indicate that such kits were protected after vaccination at the age of 4 weeks as well as at later time All the kits in this group were clinically well during the experiment

Thus in the case of very young kits, protection seems to be obtainable either by maternal antibodies or by vaccination, a combination is apparently less favorable The antibody levels do not follow the levels of protection Why the kits of vaccinated mothers would have higher titres than the ones of unvaccinated mothers is not explained by the experimental results

DISCUSSION

Unfortunately, all groups of animals used in the experiments discussed in the present report are quite small and nothing but tentative conclusions may therefore be drawn on this basis The results point towards the possibility that actively and passively acquired antibodies may be of little use or even of negative effect in the protection against distemper virus The only exception seems to be the protection observed in cases of 4 weeks

TABLE 4 *A Comparison between Vaccination of Kits from Vaccinated and not Vaccinated Mothers*

Age of kits at vacination	Kits from vaccinated mothers		Kits from not vaccinated mothers	
	Average of antibody titres at challenge	Mortality	Average of antibody titres at challenge	Mortality
4 weeks	300	0/6* = 0 %	310	0/6 = 0 %
6 weeks	2500	1/6 = 17 %	300	0/6 = 0 %
8 weeks	1000	1/8§ = 13 %	270	0/6 = 0 %
10 weeks	1300	1/6 = 17 %	450	0/6 = 0 %
No vaccination			<10	3/9 = 33 %

* All animals clinically ill with distemper

§ One animal clinically ill with distemper

old kits of vaccinated mothers but kits of unvaccinated mothers could be fully protected by vaccination at this age

The results of previous experiments (*Hansen* (1971)) in which two types of vaccines were used showed that vaccinations giving the same antibody levels may or may not give protection In the present report it was confirmed that antigenicity need not be a parameter for protection, but the levels of antibody were on a average one 5 fold dilution step lower in the non protected animals As the serum dilution steps employed in the above quoted paper were 10 fold there is not necessarily any difference in the two results The results of the two experiments might have been identical if the same dilution steps had been used This difference seems of little consequence as the titres obtained usually would be considered sufficient for protection even in the lower range (e g *Ackermann* (1966) and *Wellcome Foundation* (1970))

In the present experiment extremes in unsuitable treatment of a live vaccine were attempted but even so antigenicity was preserved The vaccines had originally good titres of protective capacity (*Hansen et al* (1973)) if properly stored Lyophilized vaccines from different producers have different stability but even the most stable ones should be stored carefully as antigenicity alone apparently is not sufficient to ensure protection

In outbreaks of distemper in mink farms at different times of the year, a number of practical problems concerning vaccination arise If an outbreak is starting in spring time the pregnant mink may present a problem but it seems that available vaccines may be safely used without causing harm to the mothers or the offspring (*Hagen et al* (1970)) Antibody may be transferred to the kits up to 2 weeks after partus (*Porter* (1965)) In outbreaks in June when the kits are between 4 and 8 weeks of age it has been recommended to use half doses of vaccines and then follow up with vaccination 2 3 weeks later using full dose (*Anon* (1967))

It has also been suggested (*Veterinærdirektoratet* (1969)) to postpone vaccinations until 14–15 weeks later because results of earlier vaccinations have been reported to be poor In the case of distemper in dogs *Baker et al* (1969) have set up a nomograph for a correlation between the antibody titres in the mothers and the optimal time for vaccinations of the puppies

In view of the results of the present work, it may not always be advisable to act according to the above quoted procedures Vaccinations of young animals using a live vaccine may apparently be successful if applied at a very early age (4 weeks) provided that the mothers have not been vaccinated and the dose is sufficient for take On the other hand maternal antibodies may protect 4 weeks old kits and may give some protection even in older individuals These antibodies should probably not be interfered with by vaccination From about the 11th week circulating antibodies alone apparently do not interfere either with live vaccine virus or virulent virus These results correspond well with the ones obtained by *Farrell et al* (1971) who report that young ferrets born of distemper immune females were responsive when they were 10 weeks old or above Thus vaccination may be carried out if desired at this age irrespective of the vaccination status of the mothers

It is interesting to note that in a small scale trial of oral poliovaccine in infants (*Faxen et al* (1962)), no 'take was obtained in very young infants who had high titres of maternal antibodies whereas the same titres in older infants did not interfere with vaccine take (as measured by an excretion period of virus and rise in antibody titre) In view of the results of this work and of the one by Faxen et al it seems that immunity may be acquired in a young individual but it has nothing to do with circulating neutralizing antibodies On the contrary these may interfere with the immunization process

The route of application is often discussed in connection with challenge experiments and

vaccinations. In works with distemper virus it does not seem to be of importance whether the virus is applied by contact, by aerosol spray, by intranasal installation or by subcutaneous injections provided the dose is sufficient for take (*Momberg Jorgensen* (1950), *Hansen et al.* (1973)).

It may be difficult to predetermine the proper dose of virulent virus in a challenge experiment. If the object of a challenge experiment is the titration of a vaccine, it may be suitable and even preferable to use a high dose of virulent virus in order that a 100 per cent mortality may be obtained in the control group. In this way even small experiments may give clearcut results and a good safety factor in the testing of the vaccine is obtained. On the other hand, if the object is a study like the present one, it is of considerable importance to approach as closely as possible a "natural" dose. As judged from epidemiological evidence this would mean that a dose giving a mortality ranging between 10–75 per cent should be chosen. As the mortality may depend on age and genetic factors and as virus titrations involve considerable experimental errors the proper dose is not easily predetermined and large scale experiments are desirable.

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HEPATITIS ASSOCIATED ANTIGEN (HAA) IN URINE FROM SERUM HAA POSITIVE RENAL TRANSPLANT RECIPIENTS

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Urine was examined for the presence of hepatitis associated antigen (HAA) by means of gel diffusion and immunoelectrophoresis in thirteen renal transplant recipients who were known serum carriers of HAA. Three 24-hour urine collections were obtained from twelve of the patients and two from one. All unconcentrated urine specimens examined were HAA negative. After 500 fold concentration HAA could be demonstrated in the urinary samples obtained from four of the patients. HAA urinary excretion was not related to any of the kidney or liver parameters studied. Antigenuria could not be explained by increased glomerular permeability suggesting that HAA may propagate in the renal parenchyma and/or in the urinary tract.

The generally accepted view that serum hepatitis is only transmitted parenterally through blood or blood products was challenged by *Giles et al.* (5) and by *Krugman et al.* (8) who experimentally induced serum hepatitis by administration of infectious serum by mouth, furthermore secondary cases were observed in some of the contacts.

Recently, several papers have reported on the detection of hepatitis associated antigen (HAA) in urine (1, 3, 4, 14). The authors suggest that urine must be regarded as infective as well as blood and serum and this may explain the occurrence of secondary cases. However, *Shulman* (11) was unable to detect HAA in urine even when concentrated a hundredfold.

Infected renal transplant recipients seem to remain HAA serum carriers with high titres indefinitely (13). The object of this study was to investigate whether HAA could be detected in urine from renal transplant recipients who were known carriers of HAA.

MATERIAL AND METHODS

The series comprised thirteen HAA positive renal transplant patients (Table 1). Transplantation had been performed in these cases between eleven and thirty eight months before our first urine examination. The primary disease was chronic glomerulonephritis in seven patients and non immunological kidney diseases in the others. Eight patients received kidneys from living related donors, the remaining five were transplanted with a necro kidney. Bilateral nephrectomy was performed on all patients. After transplantation the patients received immunosuppressive treatment with prednisone and azathioprine. The dose given during the period covered by the study appears from Table 1, in two patients, prednisone therapy had been withdrawn.

Graft function. The graft function was routinely controlled by determination of the 24 hour endo-

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TABLE 1 *Graft Function, Immunosuppressive Treatment, Serum HAA*

Case No	Sex	Age	Endogenous creatinine clearance ml/min			Proteinuria g/24 h		
2	F	47	45	42	43	0	0	0
4	M	35	77	66	82	0	0	0
5	M	38	65	59	42	1 5	3 5	3
11	M	44	53	46	52	0	0	0
16	M	31	29	31	29	5 2	5 0	4
17	F	19	54	–	42	0 7	–	0
20	M	23	102	108	100	0	0	0
21	F	26	84	81	76	0	0	0
22	M	34	42	51	55	0	0	0
56	M	55	64	70	75	0	0	0
61	M	50	37	30	33	0	0	0
67	M	21	52	51	74	0	0	0
84	F	38	65	58	64	0	0	0

† *Titres expressed in arbitrary units (12)*

§ Only urines concentrated 500 fold + denotes positive by gel electrophoresis × positive by immunodiffusion a m Ouchterlony (see text)

genous creatinine clearance In one of the patients (No 5), the glomerular filtration rate was deteriorating during the study owing to chronic rejection This patient and patient No 16 had pronounced proteinuria of the low selectivity type (7) and graft biopsies revealed chronic vascular rejection In all patients the urinary sediments were inconsiderable and cultures without significant growth

Liver function Serum bilirubine levels were normal in all patients Serum glutamic pyruvic transaminase (S GPT) levels were slightly elevated in ten patients and normal in three (No 2 17 and 56)

Determination of HAA in Serum

Serum HAA was detected by precipitin reaction in gel electrophoresis and quantitation performed by electrophoresis in antibody-containing agar gel *a m* Laurell as described earlier (12) The known duration of HAA positivity varied from five to forty one months

Determination of HAA in Urine

During the six month period from July to December 1971 three 24 hour urine collections were obtained from the patients at their ordinary follow up visits except from patient No 17 from whom only two samples were obtained The urine was examined neat and after 500 fold concentration by use of Carbowax® (polyethylene glycol 20 000) followed by dialysis against saline

Testing for HAA was performed by immunodiffusion and gel electrophoresis similar to the procedures used for the serum specimens In the immunodiffusion experiments, however, the wells were enlarged to contain 50 μl urine instead of 10 μl in order to increase the sensitivity The antiserum used showed identity with various reference antisera as described in a previous paper (12) Dr *P V Holland*, National Institute of Health U S A kindly identified the antiserum as specifically directed against HAA subtype a (the common antigenic determinant on the HAA particles) Identical results were obtained by repeated tests using two additional antisera against HAA The urine samples positive by gel diffusion showed identity with serum samples from the patient and with reference HAA positive sera

The concentrated urine samples tended to produce unspecific cloudy precipitations between the wells which disappeared after washing with saline for 24 hours Reading was performed after staining with 0 2 per cent comassie blue Concentrated urine samples from three serum HAA negative patients gave all negative results

RESULTS

Serum HAA Titres

The serum titres, expressed in arbitrary units (12), varied from 39 to 182 units in twelve out of the thirteen patients, in patient No 20 the serum concentration of HAA was too low to allow quantitation Any correlation to the dose of prednisone, the graft function, or the activity of the hepatic in

Drug Therapy (mg/24 h) Prednisone			Azathioprine			Serum HAA titres†			HAA in urine§		
10	10	10	125	125	125	118	110	105	0	0	0
0	0	0	100	100	100	102	76	86	0	0	0
10	7 5	35	100	100	75	52	85	110	+ ×	+ ×	+ ×
12.5	12 5	10	100	100	125	55	63	78	0	0	0
20	17 5	17 5	100	100	100	69	52	75	0	0	0
10	–	10	100	–	100	73	–	75	+ ×	–	+ ×
7 5	5	5	100	100	100	<3	<3	<3	0	0	0
0	0	0	125	125	125	45	77	56	×	×	+ ×
10	10	10	125	125	125	42	39	16	0	0	0
15	15	15	125	125	125	66	65	59	0	0	0
15	15	15	100	100	100	118	138	110	0	0	0
12 5	12 5	12 5	125	125	125	118	132	112	×	+ ×	+ ×
20	20	17 5	125	125	125	152	182	154	0	0	0

volvement as expressed by the S GPT levels could not be demonstrated

Urinary HAA Excretion

HAA could not be detected in any of the unconcentrated urine specimens This was in agreement with a pilot study performed during March and April 1971 in which we were unable to detect HAA in neat and 20-fold concentrated urine samples

By means of gel electrophoresis HAA could be detected in eight out of the 38 500 fold concentrated urinary specimens The positive samples originated from four patients (Table 1) Only two urine samples, obtained from patient No 5 were positive by the Ouchterlony gel diffusion technique using the ordinary 10 μl of urine in the wells However if the wells contained 50 μl, all eleven samples from these four patients were found to be positive for HAA The others remained negative One of the positive patients had pronounced proteinuria, however, HAA could not be detected in the urine from the other patient with pronounced proteinuria (No 16)

Any correlation to the primary kidney disease the source of the donor kidney, the immunosuppressive treatment, the graft function, the serum HAA titres of the S GPT levels could not be revealed

DISCUSSION

Thirty eight 24 hour urine collections from thirteen renal transplant patients, who were serum HAA carriers, were investigated for the presense of HAA HAA could be detected in 500-fold concentrated urine specimens obtained from four out of the thirteen patients The concentrations of HAA were very low

In all patients except one, the serum titres were high, presumably due to the immunosuppressive treatment However, the urinary excretion of HAA was not related to the serum titres or to the theraphy One of the patients with antigenuria did not receive prednisone

The HAA particle is so large (20 nm) that urinary excretion hardly can be explained on the basis of increased glomerular permeability This is substantiated by our study Two of the patients with antigenuria did not have proteinuria and HAA could not be detected in the urine from one of the patients with pronounced proteinuria although the

slope of the selectivity line indicated glomerular permeability to large protein molecules According to the present results it is thus unlikely that HAA is *passively excreted from* the blood stream by glomerular filtration and it is therefore suggested that the virus may propagate in the urinary tract If so, this might also explain the interesting finding by *Sherlock and co workers* (1) who reported a high concentration of HAA in the urine from their antibody donor Viruria in the presence of serum neutralizing antibody is known from cytomegalovirus disease (6)

By means of the complement fixation test, *Blainey et al* (3) could demonstrate HAA in the urine from all of seven renal transplant patients who were chronic HAA carriers If applied to sera the complement fixation test *is much more sensitive than the gel electro*phoresis technique used in our study However *Apostolov et al* (1) found that the frequency of positive reactions on urines obtained by means of gel electrophoresis was about 30 per cent higher than that obtained with the complement fixation test this indicates that gel electrophoresis seems to be more sensitive than the complement fixation test if applied to urine All the urines tested were negative by the Ouchterlony technique thus *indicating a very low concentration of* HAA Only one of the urines examined by *Blainey et al* was positive if tested by the electrophoresis technique, another was positive *only if tested by diffusion* and not by electrophoresis The discrepancies between these two papers suggest the need for further investigation

MacCallum & Bradley (9) and *Neefe et al* (10) were unable to induce serum hepatitis in volunteers by oral administration of urine The detection of HAA in urine does not necessarily imply that urine is infectious First, it is not known whether HAA detected in urine represents the complete virus or *only* the capsid which constitutes the majority of the HAA particles detected in the blood Secondly, induction of the disease is related to the dose of infectious virus (11) and the concentration of HAA *in urine is very low*

With the exception of direct handling of blood specimens by hospital personnel it is generally held that serum hepatitis is little *contagious In our material this view* is supported by the fact that only one of our transplant recipients has caused a secondary case to arise outside the department The fiancee of patient No 22 developed a HAA positive hepatitis three months after they had become acquainted HAA could not be detected in the urine of patient No 22 On the other hand, two medical officers, one laboratory technician and one nurse have contracted hepatitis, they were presumably infected by direct contact with blood from HAA positive renal transplant recipients

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TABLET SENSITIVITY TESTING: A COMPARISON OF DIFFERENT METHODS

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Regression lines and their equations have been prepared for Neo Sensitabs® containing 26 different antimicrobials using two different agar media and three different methods usual without pre diffusion 1 hour pre difusion and 1+20 hour pre diffusion The numerical value of the slope of a regression line indicates in mm the mean change in zone diameter for a 2 fold change in MIC The numerical value of the slope is increased when pre diffusion methods are employed, reaching its maximum with 1+20 hour pre diffusion method When comparing two agar media Mueller Hinton and Danish Blood Agar, the difference between the slopes of the regression lines will be only slight if the same anti microbial and the same method are used There is a linear correlation between on a loga rithmic scale the molecular weight of the antimicrobials contained in tablets or discs and on a linear scale the slope of the regression line pertaining to the particular drug

Sensitivity testing by the tablet method (Neo Sensitabs®) has been used for several years, especially for tests in primary culture (*Lund et al* 1951, *Gylling Pedersen* & *Casals* 1967) In the years up to 1969 tablets were used only in connection with the usual diffusion method, but in recent years pre-diffusion methods have aroused increasing interest among bacteriologists By the latter method the tablets are left for diffusion on the plate and then removed before inoculation takes place The two pre diffusion methods we will refer to are the 1-hour pre diffusion method and the 1+20 hour pre diffusion method

By the usual diffusion method, antibiotics are added to the medium in the form of a tablet or a disc when the incubation period starts Consequently the growth of the micro organisms and the diffusion of the antibiotic start simultaneously

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The pre-diffusion aims at separating the diffusion and the growth processes to the effect that antibiotics in tablets or discs are allowed to diffuse into the medium prior to inoculation and incubation The depot of active substance is thus removed from the agar plate, resulting in a reduction of the central concentration and an increase of the distance between 2 fixed concentrations, be cause the concentration of antibiotic at the same time increases at the periphery This reduces the possibilities of relatively resistant slow-growing bacteria being determined as more sensitive than is actually the case and, at the same time, no zones will develop with resistant strains

A period of pre diffusion after inoculation has been used by some authors in order to improve the correlation between the MIC and the diameters of the inhibition zones *Barber* & *Whitehead* (1949) placed the plates in the refrigerator for 48 hours *Klein* (1953) left penicillin to diffuse for 5 hours before incubation of the plate and found an im

proved correlation *Ericsson et al* (1954) inoculated the plates placed the discs containing antimicrobials on them and thereafter incubated for 3 hours at room temperature before the final incubation at 37° C *Hermann* (1958) using pre diffusion in the refrigerator for 48 hours obtained increased inhibition zones with the sensitive microorganisms and smaller inhibition zones with the resistant ones *Frolund Thomsen* (1962) using pre diffusion before inoculation correlates the dilution method to the diffusion method and concludes that the correlation between the two methods improves parallel to the duration of the pre diffusion *Bauer* (1964) refrigerated the plates for 2 hours before transferring them to the incubator and found that the zones became larger *Bourgeou et al* (1965) inoculated plates and determined the effect of leaving the plates at room temperature after addition of antibiotic discs for varying periods of time prior to incubation at 37° C The authors conclude that under the experimental conditions provided, pre incubation at room temperature did not further increase zone sizes to a significant extent.

The purpose of this paper is to study the three above mentioned methods (the usual without pre diffusion, 1 hour pre diffusion and 1+20-hour pre diffusion) with tablets especially by comparing regression lines and equations obtained by the different methods

MATERIAL AND METHODS

A regression line is a graphic expression of a correlation between the Minimal Inhibitory Concentration (MIC) and the inhibition zone diameters of a number of strains obtained with an antibiotic contained in a tablet or disc

We have been using the agar dilution method to determine the MIC To successive portions of a culture medium increasing quantities (twofold) of antimicrobial are added the resulting antimicrobial/agar media being poured into a series of petri dishes (plates) The surface area of each plate is then divided into 16 sections each of which is inoculated with a loopful of a standardized suspension of an overnight culture of the strains to be tested Thereafter the plates are incubated at 37° C overnight and the growth in each section of each plate is compared with the same strains in a control plate containing the medium free of antimicrobial

Using the same culture medium a series of sensitivity test plates are prepared and inoculated with the same strains and standardized inoculum as used in the MIC determination The antibiotic containing tablets are now placed on the surface of the plates and diameters of the inhibition zones are then measured after incubation at 37° C for approximately 18 hours (usual agar diffusion method of sensitivity testing) By the 1 hour pre-diffusion method the same technique is used but the tablets are placed on the plates before inoculation and allowed to remain there for one hour at room temperature The tablets are then removed and the plate inoculated and incubated By the 1+20 hour pre diffusion method the tablets are removed after 1 hour and the plates are then allowed to remain at room temperature for 20 hours before inoculation and incubation

When values of MIC and diameters of inhibition zones have been established for a sufficient number of different bacterial strains with varying degrees of sensitivity the regression lines can be drawn as a graphic linear average of the plots

A total of 128 different bacterial strains including the most current pathogens (19) have been used in the construction of the regression lines

Culture media Two different media were used 1) Danish Blood Agar (DBA) consisting of 1 per cent beef extract 1 5 per cent good quality agar 1 0 per cent dextrose 0 3 per cent sodium chloride 0 2 per cent secondary sodium phosphate (dodecahydrate) and 5 per cent defibrinated horse blood 2) Mueller Hinton Agar Medium Difco (MH) with no blood added

The thickness of layer averaging 5 mm (DBA) or 4 mm (MH)

The equation of a regression line is of the type $x = -ky + M$ in which M denotes the intersection point between the line and the abscissa k denoting the slope of the regression line Because MIC values mostly consist of fractions we have for reasons of clearness used the numerals 0 1 2 3 4 for the computation of the equations instead of the values 0 003 mcg/ml 0 006 mcg/ml 0 012 mcg/ml 0 025 mcg/ml 0 05 mcg/ml as the numerals 0 1 2 3 4 may be transferred to the MIC using the formula $\log_2$ MIC + constant = numeral

RESULTS

Figures 1 2 and 3 show regression lines referring to the 3 methods covering 6 antimicrobials using two different culture media

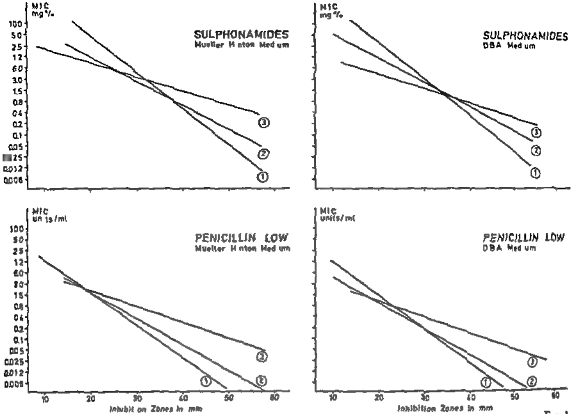

MIC mg %
SULPHONAMIDES
Mueller Hinton Medium
SULPHONAMIDES
DBA Medium
MIC units/ml
PENICILLIN LOW
Mueller Hinton Medium
PENICILLIN LOW
DBA Medium
Inhibition Zones in mm

Fig 1

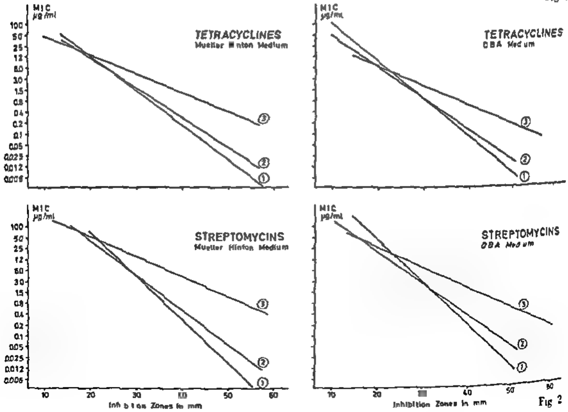

MIC µg/ml
TETRACYCLINES
Mueller Hinton Medium
TETRACYCLINES
DBA Medium
STREPTOMYCINS
Mueller Hinton Medium
STREPTOMYCINS
DBA Medium
Inhibition Zones in mm

Fig 2

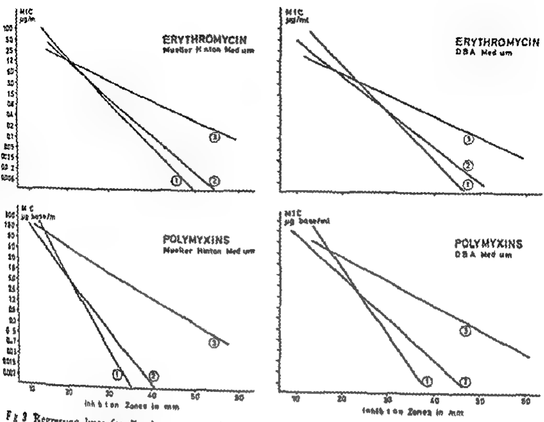


Fig 3 Regression lines for Erythromycin and Polymyxins Neo-Sensitabs®
(1) usual diffusion method
(2) 1-hour pre-diffusion method
(3) 1 + 20-hour pre-diffusion method

The figures show that 1) employing the pre-diffusion methods flatter regression lines are obtained regardless of antimicrobial 2) the regression lines of the same antimicrobial with the two different agar media have practically the same slope and 3) the lower the molecular weight of an antimicrobial the flatter the corresponding regression line

Table 1 shows equations for regression lines covering 26 antimicrobials, using the two agar media and the three different methods referred to The equations clearly show that 1) the numerical value of the coefficient of slope is increased if pre diffusion methods are employed reaching its maximum with the 1 + 20 hour pre diffusion method 2) the numerical value of the coefficient of slope is being reduced in accordance with increased molecular weights of the various antimicrobials used and 3) for most antimicrobials there is only a slight difference of slope whether DBA or MH is used

Figure 4 will provide an impression of the relationship between the slopes of regression lines and the molecular weights of various antimicrobials using Neo-Sensitabs and Mueller Hinton culture medium It is noted that there is a linear correlation between mole

Fig 1 Regression lines for Sulphonamides and Penicillin Low Neo-Sensitabs ®
(1) usual diffusion method
(2) 1 hour pre diffusion method
(3) 1 + 20 hour pre-diffusion method

Fig 2 Regression lines for Tetracyclines and Streptomycins Neo-Sensitabs ®
(1) usual diffusion method
(2) 1 hour pre-diffusion method
(3) 1 + 20-hour pre-diffusion method

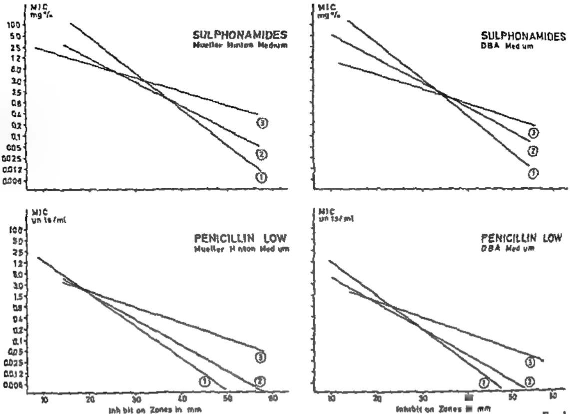
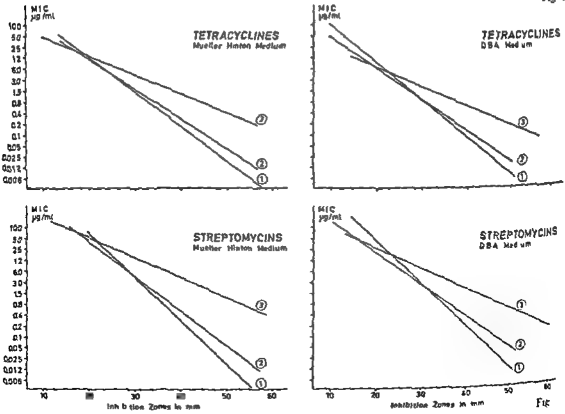

MIC mg %
SULPHONAMIDES
Mueller Hinton Medium
MIC mg %
SULPHONAMIDES
DBA Medium
MIC units/ml
PENICILLIN LOW
Mueller Hinton Medium
Inhibition Zones in mm
MIC units/ml
PENICILLIN LOW
DBA Medium
Inhibition Zones in mm

Fig 1


MIC µg/ml
TETRACYCLINES
Mueller Hinton Medium
MIC µg/ml
TETRACYCLINES
DBA Medium
MIC µg/ml
STREPTOMYCINS
Mueller Hinton Medium
Inhibition Zones in mm
MIC µg/ml
STREPTOMYCINS
DBA Medium
Inhibition Zones in mm

Fig

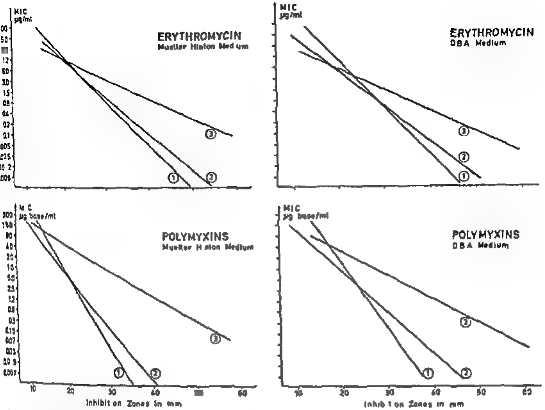


Fig 3 Regression lines for Erythromycin and Polymyxins Neo-Sensitabs®
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(3) 1+20 hour pre diffusion method

Fig 2 Regression lines for Tetracyclines and Streptomycins Neo-Sensitabs®
(1) usual diffusion method
(2) 1 hour pre-diffusion method
(3) 1+20 hour pre-diffusion method

TABLE 1 *Equations for Regression Lines with Neo Sensitabs Containing Different Antimicrobials*

Antimicrobial	Mol * wt	Culture medium	Usual diffusion method	1 h pre diffusion method	1 + 20 h pre diff method
Nitrofurantoin	238	DBA	x=-3.55y+72.2	x=-4.62y+84.0	
		MH	x=-3.51y+70.1	x=-4.60y+81.8	
Nalidixan	254	DBA	x=-3.27y+63.5	x=-3.63y+66.5	
		MH	x=-3.40y+65.8	x=-4.00y+73.0	
Sulphonamides	270	DBA	x=-2.98y+59.8	x=-4.65y+75.2	x=-7.50y+ 9
		MH	x=-3.05y+62.3	x=-4.62y+75.0	x=-7.71y+10
Trimethoprim	290	DBA	x=-3.41y+55.5	x=-4.71y+62.4	
		MH	x=-3.67y+58.0	x=-4.38y+60.1	
Trimethoprim + Sulfa	290/270	DBA	x=-3.45y+63.4	x=-5.74y+76.2	
		MH	x=-3.16y+60.8	x=-4.52y+71.0	
Chloramphenicol	323	DBA	x=-3.59y+67.5	x=-4.71y+78.4	x=-7.44y+11
		MH	x= 3.38y+65.0	x=-4.06y+71.7	x=-7.48y+11
Ampicillin	349	DBA	x=-3.07y+55.8	x=-3.97y+62.8	x=-6.56y+ 9
		MH	x=-3.44y+59.8	x=-4.08y+65.6	x=-6.66y+ 9
Penicillin Low	356	DBA	x=-3.17y+48.0	x=-4.31y+55.2	x=-6.85y+ 7
		MH	x=-3.21y+50.0	x=-4.14y+57.6	x=-6.72y+ [illegible]
Penicillin High	356	DBA	x=-3.11y+57.8	x=-3.61y+61.7	
		MH	x= 3.06y+59.8	x=-3.80y+64.0	
Cephalosporins	418	DBA	x= 2.92y+55.3	x=-3.58y+61.2	x=-6.67y+89
		MH	x=-2.74y+56.6	x=-3.33y+60.2	x=-6.53y+94
Methicillin	420	DBA	x=-3.17y+56.2	x=-4.05y+61.8	x=-6.00y+83
		MH	x=-3.28y+60.8	x=-4.14y+67.4	x=-6.05y+89
Carbenicillin	422	DBA	x=-3.56y+64.9	x=-4.23y+69.8	
		MH	x=-3.08y+62.0	x=-3.70y+66.4	
Lincomycin	452	DBA	x= 3.69y+63.5	x=-3.91y+65.4	
		MH	x=-3.46y+59.5	x=-3.94y+63.3	
Tetracyclines	497	DBA	x=-2.92y+53.6	x=-3.50y+59.0	x=-5.66y+83
		MH	x=-3.07y+57.4	x=-3.73y+64.4	x=-5.72y+90
Virginiycin	–	DBA	x=-2.58y+50.6	x=-3.14y+53.1	
		MH	x=-3.18y+60.8	x=-4.00y+67.0	
Fucidin	539	DBA	x=-2.07y+48.4	x=-2.84y+56.9	
		MH	x=-2.63y+58.8	x=-3.21y+65.0	
Kanamycin	601	DBA	x= 2.35y+50.8	x=-3.17y+59.7	x=-4.86y+81
		MH	x=-2.73y+58.9	x=-3.13y+61.8	x=-4.92y+89
Novobiocin	650	DBA	x=-1.82y+39.2	x=-2.42y+43.2	
		MH	x=-2.41y+48.8	x=-2.99y+54.5	
Gentamicin	710	DBA	x=-1.99y+46.4	x=-2.86y+54.7	x=-5.00y+76
		MH	x=-2.33y+51.0	x=-2.80y+54.0	x=-4.76y+80
Streptomycins	731	DBA	x=-2.38y+55.4	x=-5.25y+62.5	x=-5.31y+88
		MH	x=-2.43y+55.3	x=-3.14y+63.1	x= 5.27y+95
Rifamycins	823	DBA	x=-2.60y+50.0	x=-3.27y+54.6	
		MH	x=-2.70y+48.4	x=-3.33y+54.3	
Erythromycin	873	DBA	x=-2.43y+47.7	x= 3.05y+53.0	x=-5.17y+77
		MH	x=-2.40y+49.5	x=-2.86y+54.0	x=-5.19y+82
Neomycin	1167	DBA	x=-1.91y+42.8	x=-2.87y+51.8	
		MH	x=-1.99y+48.6	x= 2.53y+53.4	
Polymyxins	1336	DBA	x=-1.98y+43.8	x=-2.73y+49.6	x=-4.66y+79
		MH	x=-1.43y+35.3	x= 2.00y+40.6	x=-4.17y+76
Bacitracin	1411	DBA			
		MH	x=-2.13y+46.4	x=-2.58y+49.4	
Vancomycin	3300	DBA	x=-1.62y+34.8	x=-2.55y+41.3	
		MH	x=-1.69y+37.8	x=-2.35y+43.8	

DBA = Danish Blood Agar Culture Medium MH = Mueller Hinton Culture Medium

* The molecular weight refers to the salt of antimicrobial contained in the tablet

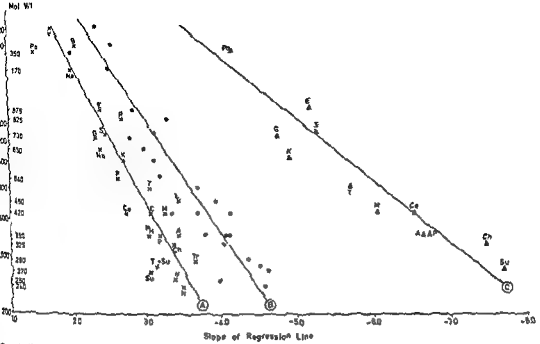

Fig 4 Relationship between slopes of regression lines and molecular weights of antimicrobials
(A) usual diffusion method
(B) 1 hour pre-diffusion method (C) 1 + 20 hour pre diffusion method
A = Ampicillin, B = Bacitracin, C = Carbenicillin, Ce = Cephalosporins Ch = Chloramphenicol, E = Erythromycin, F = Fucidin, G = Gentamicin K = Kanamycin L = Lincomycin, M = Methicillin N = Nalidixan, Ne = Neomycin, Ni = Nitrofurantoin, No = Novobiocin, P = Penicillin Low, PH Penicillin High, Po = Polymyxins, R = Rifamycins, S = Streptomycins, Su = Sulphonamides, T = Tetracyclines, Tr = Trimethoprim, Tr+Su = Trimethoprim + Sulphonamides, V = Vancomycin

cular weights on a logarithmic scale and slope on a linear scale Moreover a separation of the antimicrobials into three groupings of molecular weights is noted, the lower ranging between 240 and 500, the central ranging between 500 and 900 and the higher grouping having molecular weights beyond 1000

As might be expected, the lines representing the pre diffusion methods (B and C) are distinctive from the line representing the usual diffusion method (A), and a computation reveals that, as an average for all antimicrobials the numerical slope value by the 1 hour pre-diffusion method is about 0 65 units higher than the value obtained by the usual diffusion method In a similar manner, by 1+20 hour pre diffusion we obtain a numerical slope value which is about 2 8 units higher than the corresponding value, obtained by the method without pre-diffusion

This, in turn, means as we will show later that on an average the distance between two subsequent MIC-values is 0 65 mm and 2 8 mm higher when the two pre-diffusion methods referred to are employed, as compared to the method without pre diffusion

DISCUSSION

Several authors have been using regression lines permitting a quantitative interpretation from inhibition zone sizes over a range of MIC's for antimicrobials Recently, *Matsen et al* (1969) prepared regression lines for several antimicrobials using the Kirby Bauer method *Washington* & *Yu* (1970) prepared regression lines for cephalothin, cephaloridine and cephalexin in Mueller Hinton Agar and Trypticase Soy Agar and found that regression lines with both media were practically

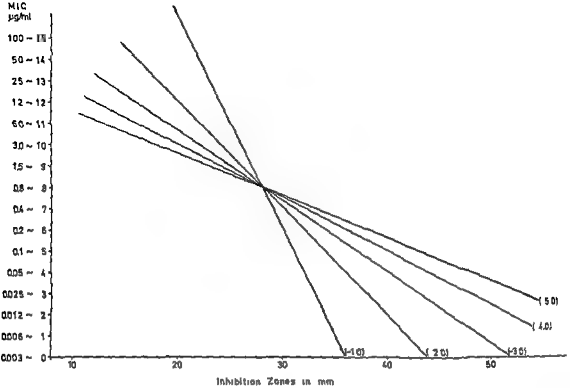

Fig 5 Regression lines with varying slopes

superimposable The International Collaborative Study under W H O (1971) have prepared regression lines for 10 different anti microbials using MH medium

Frolund Thomsen (1967) has prepared regression lines for 15 antimicrobials by a disc method with 20 hours pre-diffusion with DBA medium containing 10 per cent horse blood *Rosdahl & Frolund Thomsen* (1971) have recently prepared regression lines for carbenicillin and gentamicin by the same method

As mentioned already, K in the equation $x = -Ky + M$ represents the slope of a regression line The higher the numerical value of K, the flatter the regression line

Figure 5 shows regression lines with varying slopes, i e K-values ranging from 1 0 to 5 0

On closer inspection of the line presenting the slope −1 0, a difference in inhibition zones of 1 mm between two subsequent MIC-values will be seen Accordingly, for lines with the slope −2,0, −3 0, −4 0 and −5 0 the differences between two subsequent MIC-values are 2, 3, 4 and 5 mm This means that the numerical value of the slope of a regression line is immediately convertible into a distance in mm between two subsequent MIC values and the longer the distance, the safer the sensitivity testing will be, because the factors that may affect the size of the inhibition zone will be less influential

As shown in table 1, there is a clear correlation between the molecular weight of an antimicrobial and the slope of the regression line pertaining to the particular substance This is not surprising because they are mainly the physico-chemical properties of a substance which determine the diffusion in a solid medium, meaning that an anti microbial of low molecular weight will diffuse more easily into an agar medium than an antimicrobial of high molecular weight

With most antimicrobials it is also to be expected that the diffusion in an agar medium will be largely independent of the composition of the culture medium The varia-

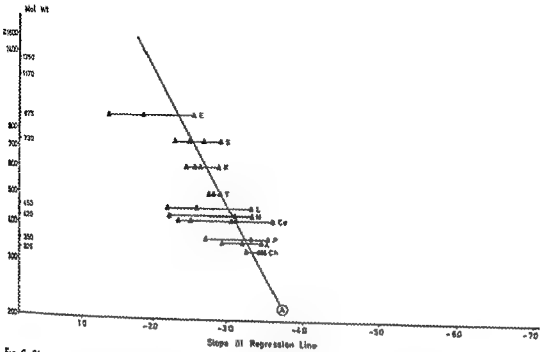

Fig 6 Slopes of regression lines obtained in 7 different laboratories (usual diffusion method) A = Ampicillin, Ce = Cephalosporins, Ch = Chloramphenicol, E = Erythromycin, K = Kanamycin L = Lincomycin M = Methicillin, P = Penicillin, S = Streptomycins T = Tetracyclines

tions found in the slope of the regression lines with certain antimicrobials in connection with different agar media may be mainly due to variations in the agar used, and specially its content of ions interfering with those antimicrobials

Several authors have studied the interference between agar and antimicrobials *Hanus et al* (1967), *Kunin & Edmonson* (1968) and *El Nakeeb & Yousef* (1970) have reported that the aminoglycoside antibiotics and the polymyxins are bound to agar

The effects of salts in the agar media on the diffusion and activity of aminoglycoside type antibiotics is well documented, *Oden et al* (1963)

Garrod & Waterworth (1969) found that the diameter of inhibition zones in cultures of *Pseudomonas aeruginosa* with discs of gentamicin varied on different media, as did the MIC by the agar dilution method using the same medium The phenomenon was found to be due to differing concentrations of Mg^{++} in the broth bases and in the agars

These results were confirmed by *Traub* (1970) using MH Broth and MH Agar

Raymond & Traub (1971) obtained similar results with gentamicin and enterococcal isolates

It is also well known that some media reduce the apparent activity of tetracyclines because of their Ca^{++} and Mg^{++} contents (*Jay & Sherris* 1971) and that addition of whole or lysed blood greatly reduces the activity of highly protein bound antibiotics as fucidin and novobiocin (*Garrod & Waterworth* 1971)

The mentioned studies concerning interferences between antimicrobials and agar ions or protein-binding indicated in most cases that a variation in the size of the inhibition zone, because of use of a certain type of agar or addition of salts to the agar medium, is accompanied by a change in the MIC value Increase of the zone size is accompanied by a decrease in the MIC value and *vice versa* These results must be interpreted to the effect that these changes in the com

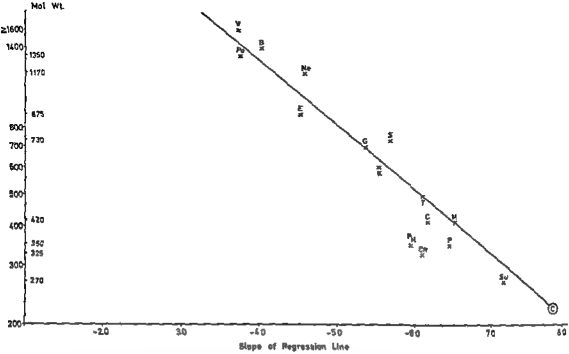

Fig 7 Slopes of regression lines covering 14 antimicrobials (20 hour pre diffusion method) B = Bacitracin C = Carbenicillin, Ch = Chloramphenicol, E = Erythromycin, G = Gentamicin, K = Kanamycin M = Methicillin, Ne = Neomycin, P = Penicillin Low, P_H = Penicillin High, Po = Polymyxins S = Streptomycins Su = Sulphonamides T = Tetracyclines

position of the medium do not alter essentially the slope of the regression line Only in case of antimicrobials highly bound to some types of agar (polymyxins, some aminoglycosides) or highly proteinbound antibiotics (fucidin, novobiocin) the latter in connection with blood agar media, one may expect a more significant variation in the slope of the regression lines

Our investigations have shown that it applies to most antimicrobials that 5–6 mm larger inhibition zones have been obtained using MH compared to DBA, but we have also found insignificant differences in the slopes of the regression lines

In Figure 6 are shown slopes of regression lines covering 10 antimicrobials (discs containing ampicillin, cephalothin chloramphenicol, erythromycin, kanamycin, lincomycin methicillin penicillin streptomycin and tetracycline) by the usual diffusion method, obtained in 7 different laboratories in connection with WHOs International Collaborative Study on Antibiotic Sensitivity Testing (1971)

The full line is identical to the one drawn in Figure 4 (line A) which showed the correlation between slope and molecular weight in connection with the use of tablets by the method without pre diffusion, and it is shown that this line is the expression of a fair average of the results encountered if discs are used

In Figure 7 are shown slopes of regression lines covering 14 antimicrobials (discs containing bacitracin, carbenicillin, chloramphenicol, colimycin, erythromycin, gentamicin kanamycin, methicillin, neomycin, penicillin, streptomycin, sulfathiazol, tetracycline and vancomycin) using the 20 hour pre diffusion method and DBA medium containing 10 per cent horse blood The equations of the regression lines have been calculated according to results described by *Frolund Thomsen* (1967) and *Rosdahl* & *Frolund Thomsen* (1971) The full line is identical to the one

drawn in Figure 4 (line C) which showed the correlation between slope and molecular weight using tablets and the 1+20-hour prediffusion method

We consider that the diffusion of antimicrobials in a solid medium is independent of excipients, whether paper or tablet excipients Our results also show that the slope of a regression line must be largely independent of the amount of active substance in a disc or in a tablet as it is possible to obtain the same slope and thus identical or parallel gradients of concentration using discs containing a small amount of an antimicrobial or tablets containing a higher amount

Our experiments have shown that it applies to the three diffusion methods studied that there is a clear correlation between the molecular weight of the antimicrobials contained in tablets or discs and the slope of the regression line pertaining to the particular antibiotic, this assumption is valid for both of the media tested and probably will be valid for most of the culture media used for sensitivity testing

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SOME CHARACTERISTICS OF THE A ANTIGEN IN MAREK'S DISEASE VIRUS—INFECTED CELL CULTURES

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The A antigen in Marek's Disease Virus infected cell cultures was found to be stable at 37° C or lower for long periods to be rapidly inactivated at temperatures at 75° C to 100° C unaffected by ether or chloroform tolerate pH 1 7 for 4½ hours but is inactivated by pH 12 1 in 4½ hours Trypsin inactivates the antigen activity in 40 60 minutes at 37° C and it remains in the supernatant after centrifugation at 100 000 × G for 120 minutes A antigen preparations were found inhibitory to the replication of MDV in cell cultures and to VSV plaque formation in CK cultures but not in L cells The possibility that the A antigen is an interferon associated protein is discussed

Several antigens can be demonstrated in Marek's Disease Virus (MDV) – infected cell cultures Using the Ouchterlony technique of double difusion in agar gel *Chubb & Churchill* (1968) and *Churchill et al* (1969) found at least 6 lines under optimal conditions and the 3 major lines (A B and C) were regularly seen The A antigen was found in the medium whereas the B and C antigens were found in the cells only They also reported that the A antigen was lost on continuous passage of MDV *in vitro* No A antigen could be detected in the medium after 33rd passage and at this passage level the virus was found non oncogenic for day old chickens

This report will describe some characteristics of the A antigen

MATERIALS AND METHODS

Viruses and cell cultures MDV strain II and VII have been described earlier (*Settnes* 1970 1971) MDV referred as high passage (HP) was strain II in its 48th passage *in vitro* This HP MDV behaves as an MPA type MDV according to the terminology of *Churchill et al* (1969) and it has proved non oncogenic to day old chickens (*Settnes* 1972)

The Vesicular Stomatitis Virus (VSV) was obtained from Dr *I Rode Pedersen* as a stock preparation containing $1\,3 \times 10^5$ pfu/ml L cells are grown in Eagle's minimum essential medium (MEM) supplemented with 8 per cent inactivated calf serum and antibiotics.

Preparation of A antigen Maintenance medium (Eagle's basal medium with 1 per cent calf serum (BME 1)) from chicken kidney (CK) cultures heavily infected (more than 80 per cent of the cells infected) with low passage (i e MDV with less than 20 *in vitro* passages) MDV was centrifuged twice (3000 × G for 15 minutes and 40000 × G for 60 minutes) and the second supernatant used as A antigen For some experiments this A antigen was concentrated 10 to 20 times by precipitation with ammonium sulphate (50 per cent saturation) Precipitates were resuspended in redestilled water PBS or BME 1 The antigen preparations were stored at — 70° C

BME 1 from uninfected cultures served as negative used medium control antigen

Antisera Antibody containing sera were obtained from 5 months old convalescent birds that had survived a contact infection as day old or from convalescent birds hyperimmunized with the MDV

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strain they initially survived 4 to 6 booster injections were given at weekly intervals each injection containing 3 to 18 × 10³ pfu low passage (LP) MDV either strain II or strain VII Bleedings were performed 7 days after each injection Sera was stored at — 20° C until used These hyperimmune sera were examined for antibodies against calf serum in agar gel diffusion tests, and if positive, they were absorbed with calf serum until no reaction could be seen

Agar gel diffusion The A antigen activity was measured by the Ouchterlony technique as described by *Chubb & Churchill* (1968) Their agar formula (*Chubb* 1969) has been used throughout these studies It consists of Veronal 1 15 g Barbital Na 0 75 g NaCl 160 g NaN_3 1 g, agar (Difco Special Noble) 10 g and redistilled water ad 1000 ml pH 7 0

Termostability The A antigen activity has been studied in gel after various holding periods at temperatures ranging from 100° C to — 70° C

Sensitivity to ether and chloroform Samples of 10 times concentrated antigen in PBS (un concentrated antigen being the amount of antigen obtained in two-day old maintenance medium from heavily infected cultures) were mixed with 20 per cent (v/v) ether or chloroform and placed at 20° C for 4½ hours Samples were tested for antigen activity after evaporation of ether or chloroform

pH treatment The antigen activity was studied in gel after one hour and after 7 days at different pH s The desired pH was obtained by resuspending re precipitate pellets in Sørensen s phosphate buffer

Samples with pH 2 2 and 1 7 were obtained by adding 0 1 N HCl directly to antigen samples containing 10 times concentrated antigen in re distilled water pH 12 1 was obtained by adding 0 5 N NaOH directly to antigen samples At the end of the test period pH was brought back to neutrality by addition of HCl and NaOH

Trypsin sensitivity The technique outlined by *Bro Jorgensen* (1971) was followed 0 45 ml 10-times concentrated antigen was mixed with 0 05 ml trypsin (Trypure Novo®, 40 mg/ml) and kept at 37° C for different periods At the end of the period the mixture was placed on ice bath and 0 05 ml soy bean trypsin inhibitor (80 mg/ml) was added Mixtures were placed at 4° C until they could all be tested

Ultracentrifugation The antigen preparations were centrifuged in a Spinco centrifuge, rotor SW 50 30000 rpm (100000 × G) for 120 minutes at 4° C After the run the uppermost 0 5 ml was tested in agar gel for activity

Effect of A antigen preparations on high passage (HP) MDV 24 hour CK-cultures were drained and given new growth medium (BME-4) with different dilutions of A antigen undiluted antigen being the amount of antigen obtained in two-day old maintenance medium from heavily infected cultures the cultures were left for further incubation overnight The next day cultures were infected with HP MDV (*strain II passage 48*) in BME I medium and left for two days with the inoculum After one change of maintenance medium the cultures were read on the 4th day Foci and plaques were counted in an inverted microscope at 25 × magnification

TABLE 1 *The Stability of the A Antigen in Marek s Disease Virus Infected Cell Cultures at Various Temperatures*

np	Holding period																									
	Minutes									Hours		Days										Months				
	0	1	2	3	4	5	10	11	40	3	4	1	2	3	5	[illegible]	11	30	60	78	90	4	5	6	18	28
	+	0																								
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	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*) indicates not tested

TABLE 2 *The pH Stability of the A Antigen*

Holding period	Activity in gel of A antigen preparations at various pH s											
	1.7	2.2	2.3	3.4	4.5	5.0	5.7	6.6	7.3	7.5	9.4	12.1
1 hour			+	+	+	+	+	+	+	+	+	0
4½ hours	+	+										
7 days			+	+	+	+	+	+	+	+	+	

Effect of A antigen preparations on VSV plaque formation 0.7-0.9 × 10^6 L cells were seeded in 50 mm plastic petri dishes (Nunclon®) with 5 ml growth medium The next day they were drained and treated with A antigen dilutions for 3-4 hours as indicated in Table 5 after which they were drained and infected with VSV (approximately 50 pfu/plate in 0.5 Hanks BSS with 0.5 per cent calf serum) 60 minutes at 20° C was allowed for adsorption After draining the cultures were given 5 ml agar medium (BME-4 with 1 per cent agar) The next day a further 5 ml of agar medium was given this time including neutral red 50 ug/ml) and plaques were read the following day

VSV titration on CK cultures was performed on 3 day old cultures, and agar medium was BME-0.5 with 1 per cent agar The ensuring procedure were as those described above

RESULTS

Termostability As seen in Table 1, the A antigen activity is rather termostabile at temperatures at 37° C or lower An inactivation time has not been obtained at any of these temperatures The A antigen activity is lost in 4 days at 56° C, in 2 days at 60° C, in 4 hours at 65° C in 10 minutes at 70° C and

TABLE 3 *Trypsin-Sensitivity of the A Antigen**

	Activity of A antigen in gel after treatment with trypsin at various periods at 37° C in minutes							
	6	10	20	30	40	60	120	180
Without Trypsin	+	+	+	+	+	+	+	+
With Trypsin	+	+	+	+	+0	0	0	0

* second supernatant precipitate resuspended in PBS to give 10 times concentration

TABLE 4 *Effects of A Antigen Preparations on High Passage MDI*

A antigen* concentration or dilution	Exp I (47th pass.)		Exp II (48th pass.)		Exp III (48th pass)	
	P+F§	P F	P+F	P F	P+F	P F
×16					0	
×8					10	0.7
×4					15	0.6
×2					37	0.8
×1	184	0.27	150	1.3	28	1.2
1:2	155	0.30	128	2.1	22	1.3
1:4	136	0.47	162	2.2	26	2.1
1:8	174	0.61	172	2.0	26	3.2
1:16	117	0.57	150	3.5	19	2.6
fresh medium	178	0.62	144	3.7	27	2.9
"used medium"					25	3.8

* second supernatant precipitate resuspended in BME I giving 16 times concentration
§ P = plaque F = focus

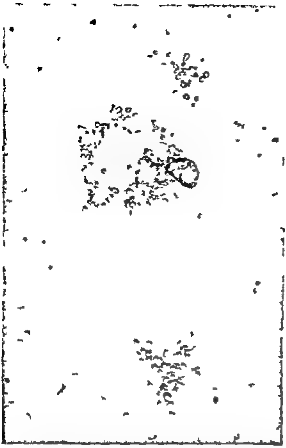

Fig 1 Cytopatic effect in chicken kidney cell monolayers 4 days after infection with high passage MDV CPRL II (48th in vitro passage) Two foci and one plaque can be seen × 100

in less than 1 minute at 75° C 80° C and 100° C

Sensitivity to ether and chloroform Ether or chloroform are without any effect on the A antigen

pH stability The results are shown in Table 2 The A antigen activity is destroyed at pH 12 1 for 4½ hours but can withstand pH 1 7 and 2 2 for 4½ hours and pH s 2 3 to 9 4 for 7 days

Trypsin sensitivity The A antigen activity is sensitive to the action of trypsin at 37° C as shown in Table 3

Ultracentrifugation The A antigen activity remained in the supernatant after centrifugation at 100000 × G at 4° C

Effect of A antigen preparations on high passage MDV The results of 3 experiments are given in Table 4 Normally the high passage MDV (strain II, 47th and 48th passage in vitro) produces macroplaques in 3–4 days if virus is given to cultures when they are established and when given to 48 hours monolayers the CPE consists of approximately ½ to ⅔ macroplaques, the rest being microplaques or foci These two kinds of CPE are shown in Figure 1

Pretreatment of CK monolayers with A antigen preparations changes the plaques over foci ratio (P F ratio) the P F ratio in treated cultures is lower than the ratio in untreated cultures The effect on P F ratio is dose related and no effect is seen on the total CPE (i a P+F) unless high concentrations of A antigen (4 to 16 times) are used (Table 4 exp III) but in these cases the growth of cells is impaired or delayed so that monolayers on the 4th day were only 50 per cent confluent and counting of foci less optimal

Effect of A antigen preparations on VSV plaque formation The result of one experi

TABLE 5 *The Effect of A Antigen Preparations* on VSV Plaque Formation in L Cells and Primary CK Cell Cultures*

Type of cell culture	Number of plaques counted antigen dilutions in medium (final) un dil	1 2	1 4	1 8	1 16	no antigen fresh medium
L cells	30§	26	33	48	38	40†
CK	4	8	11	10	13	18

* second supernatant § average of two plates † average of 5 plates

TABLE 6 *Comparative Properties of A Antigen Preparations and Interferon*

	A antigen	Interferon*
Stability at pH 2	yes	yes
Trypsin sensitivity	yes	yes
Termostability	yes	yes
Virus specificity	no	no
Cell specificity	yes	yes
Non dialysable	yes§	yes
Non sedimentable at 100 000 × G for 1 2 hours	yes	yes
Antigenicity for homologue host	yes	no

* *Fontes* (1970) § unpublished

ment is shown in Table 5 The A antigen preparation is inhibitory to the VSV plaque formation in CK monolayers but not in L cell monolayers

DISCUSSION

The results of these experiments show that A antigen induced by MDV in cell cultures is termostabile at temperatures at 37° C or lower (contrary to the virus itself (*Calnek & Adldinger* 1971)) High temperatures (75° C to 100° C) rapidly inactivate the antigen activity The antigen is unaffected by 20 per cent (v/v) ether or chloroform It tolerates pH 1 7 for 4½ hours, but is inactivated by pH 12 1 in 4½ hours Trypsinization for 40–60 minutes at 37° C inactivates the antigen activity which together with the inactivation temperatures/times indicates that we are dealing with a protein. It is rather low molecular weight protein too, as it cannot be pelleted by 100000 × G in 120 minutes (i e soluble)

All field isolates of MDV so far found induce this A antigen and produce foci only if plates are read 4 to 6 days after infection i e the plaques foci (P F) ratio is zero High passage MDV produces plaques in 4 days and no or very little (concentration 100–200 times of the medium over HP MDV infected cells gives a faint line in gels) A antigen is produced The P F ratio is high If some A antigen preparation is added to cultures before they are infected with HP-MDV, P F ratios that are lower than those in untreated cultures can be obtained The ratios move towards ratios characteristic of strains less *in vitro* adapted The A antigen preparations seem to be inhibitory to the replication of MDV in CK cultures, this inhibitory effect is also evident against another virus (VSV), but only in CK cells not in L cells

All these characteristics of the A antigen preparation, except antigenicity in the host where it is induced would tend to classify it as an interferon like substance (Table 6)

The problem therefore remains to be solved whether the A antigen is interferon itself or part of interferon although it is antigenic in chickens or whether it is an interferon like or interferon inducing protein which in most properties is very similar to interferon itself

Recently induction of interferon in vivo and in vitro with MDV has been described *Hong & Sevoian* (1971) described interferon production in chicken plasma induced with the JM strain of MDV Greatest activity was present 48 hours after inoculation, and they found higher levels of interferon in plasma from genetic resistant chickens (K line birds) than in plasma from susceptible chickens (S line birds) They found that turkey herpes virus (HVT, FC126) induced similar amounts of interferon in K and S birds

Kaleta & Bankowski (1972) described interferon production in cell cultures infected with low passage MDV (CAL 1 type 1) but not with high passage MDV (CAL 1 type 2) or with HVT strain FC126 They observed a decrease in interferon production with passage number in vitro associated with a shift in type of CPE from that characteristic of type 1 (foci or microplaques) to that characteristic of type 2 (i e macroplaques) They described an inhibitory effect on formation of foci after 10–11 days' incubation, an effect we could not see because our observation period was 4 days only

The data mentioned above, however, give no information concerning the earlier mentioned questions, as *Hong & Sevoian* (1971) do not mention the A antigen and *Mikami & Bankowski* (1971) state that they are unable to identify the A antigen

Paucker & Stanček (1972) very recently described and characterized proteins intimately associated with interferon ("Interferon associated proteins") and the A antigen could be such a protein

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LEUKOLYTIC ACTIVITY OF *ACINETOBACTER CALCOACETICUS*

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The leukolytic activity of haemolytic culture filtrates of *Acinetobacter calcoaceticus* has been investigated by a vital dye technique and by electrophoresis, employing mouse thymocytes. The leukolytic and haemolytic (phospholipase C) activities showed the same characteristic features. Both were activated by Mg^{++}, inactivated by ethylendiaminetetraacetate and heating, had similar pH optima and showed the same electrophoretic mobilities.

Bacterial haemolysins frequently show lytic activity towards a variety of mammalian cells. Leukocytes are lysed by, for example, staphylococcal α-, β- and δ-haemolysins and by streptolysins S and O (for a survey, see *Bernheimer* (1)).

In a previous paper (9), the haemolytic activity of *Acinetobacter calcoaceticus* has been described. The haemolytic and phospholipase C activities of this organism have been shown to be closely associated (10, 11).

This paper reports an investigation of the action of a culture filtrate of *A. calcoaceticus* on mouse thymocytes *in vitro*.

MATERIALS AND METHODS

The bacterial strain used (1318/69), a chemically defined growth medium and the incubation technique have been described elsewhere (9, 11). Reagents used were of analytical grade. Active culture filtrates were obtained by concentrating culture supernatants in an Amicon Ultrafiltration cell (Lexington, Mass., USA) using a Diaflo membrane (UM 20E, N_2-pressure 3 kp/cm^2).

Haemolytic activity was estimated according to the standard method (9). One haemolytic unit (H.U.) was defined as the amount of haemolysin that would produce 50 per cent lysis of 1 ml 1 per cent (v/v) human red cell suspension (pH 7.6, 60 min, 37° C).

Thymocytes were obtained from closed colony, white mice, 3–5 months old, of both sexes, killed by cervical dislocation. The thymus was removed, freed of fat and blood and pressed gently through a fine, stainless steel mesh with a siliconized rubber cork. The cells were washed twice in 145 mM NaCl and centrifuged at 150 *g* for 7 min.

Estimation of cytolytic activity. The thymocytes were suspended in 145 mM NaCl at a concentration of 1×10^7 cells/ml. Viability was 80–90 per cent. Equal volumes of cell suspension and culture filtrate were mixed and incubated at 37° C. Samples were collected at 0, 30, 60 and 90 min, and the number of degenerated cells estimated by a vital dye technique according to *Boyse et al.* (4). Freshly prepared 0.2 per cent (w/v) trypan blue dissolved in 145 mM NaCl was added to an equal volume of incubation mixture and the number of blue, degenerated cells was counted immediately. For technical reasons it was not possible to count more than one hundred cells in each sample. All experiments were performed at least three times. Experimental details are described in Fig. 1 and Fig. 2.

Electrophoresis. Crude culture supernatant was concentrated from 900 to 5 ml by ultrafiltration

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and dialyzed overnight at 4° C against 500 volumes of distilled water To obtain a sufficiently active preparation, the dialyzate was lyophilized and re dissolved in 0 2 ml 145 mM NaCl (haemolytic ac tivity 3920 H U./ml Samples of 3 µl were applied to cellulose acetate strips, 2 × 12 5 cm (Selectron Carl Schleicher & Schull Dassel West Germany) and run in 0 05 M diethylbarbiturate buffer pH 8 6 (90 min 4° C 0 4 mA/cm) in a Shandon electrophoresis unit Zones of haemolytic and of leukolytic activity were detected by applying the strips to the surface of two sets of microscope slides (2 5 × 7 5 cm), previously coated with agar (1 per cent Bacto agar, Difco Lab Detroit, Mich, USA), one set containing red cells and the other thymocytes The red cell agar (0 75 ml/slide) consisted of 1 5 per cent washed, human red cells in 0 05 M tris pH 7 6, with 130 mM NaCl and 5 mM $MgCl_2$ The thymocyte agar (0 5 ml/slide) contained 1 5 per cent washed thymocytes and 0 5 mg/ml DEAE Dextran (Pharmacia Uppsala, Sweden) suspended in Eagle medium pH 7 4 (7) Strips and slides were incubated at 37° C in 5 per cent CO_2 Haemolytic zones could be observed when the strips were removed from the slides after 10 15 min Leukolysis was visualized as recommended by *Fuji et al* (8) After 60 min incubation, the strips were removed from the thymocyte agar slides The slides were rinsed in cold PBS air-dried with a fan at room temperature and fixed in 95 per cent ethanol Photographs of haemolytic and leukolytic zones were put together to align the strips according to the points of application

RESULTS

Several samples were examined for leukolytic activity A typical experiment is shown in Fig 1 The reaction rate was found to be constant up to a level of 80–90 per cent cytolysis Mg^{++} (final concentrations 5 mM) accelerated the activity Ethylendiamine tetraacetate (5 mM Na-EDTA) and heating (65° C, 25 min) inhibited cytolysis, showing values closely corresponding to a blank that consisted of cells incubated with the buffer solution (without haemolysin)

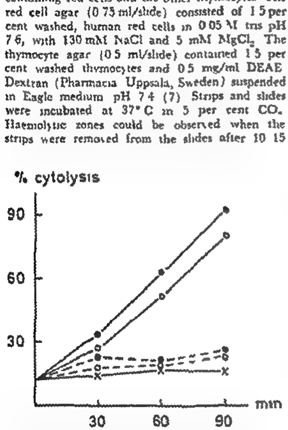

Fig 1 Influence of Mg ethylendiaminetetraacetate and heating on leukolytic activity *Culture* filtrate (22 H U /ml in 0 05 M tris pH 8 with 130 mM NaCl) was mixed with an equal volume of thymocytes suspended in 145 mM NaCl (1 × 10 cells/ml) and incubated at 37° C (O——O) (O – O) Culture filtrate heated at 65° C for 25 min before admixture (●——●) MgCl (x——x) Ethylendiaminetetraacetate added to culture filtrate in concentrations of 10 mM (● – ●) Buffer blank Cytolysis was estimated as the percentage of trypan blue—positive cells

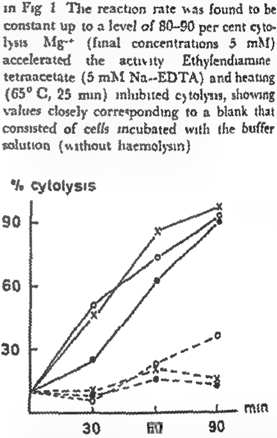

Fig 2 Influence of pH on leukolytic activity *Culture filtrates at pH 6 pH 7 and pH 8* were obtained by mixing 3 parts of culture filtrate (65 H U /ml in distilled water) with 1 part of concentrated buffer solutions Final buffer concentrations were pH 6 0 13 M phosphate buffer, with 130 mM NaCl and 10 mM $MgCl_2$ *pH 7 and pH 8* 0 05 M tris with 130 mM NaCl and 10 mM $MgCl_2$ Incubation with thymocytes and estimation of cytolysis were performed as described in Fig 1 (●——●) pH 6 (O——O) pH 7 (x——x) pH 8 Broken lines ( ) Culture *filtrates at* pH 6 pH 7 and pH 8 inactivated by heating at 65° C for 25 min

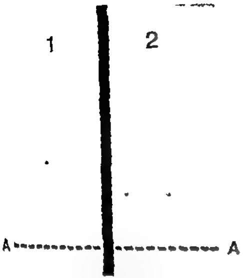

Fig 3 Leukolytic and haemolytic activities examined by electrophoresis 3 µl concentrated culture filtrate (3970 H U/ml) were applied to two

... cytes and the other strip to an agar containing human red cells.

1 Thymocyte agar after the strip has been applied upon the agar for 60 min

2 Red cell agar after the strip has been applied upon the agar for 10 min

"A" indicates the zero migration line of electrophoresis.

In repeated experiments the initial reaction rate at pH 6 was found to be lower than at pH 7 and pH 8 (Fig 2) The activities at pH 7 and pH 8 were essentially similar, but their relationship varied somewhat in different experiments Blanks consisting of cells and heat inactivated culture filtrate (pH 6 pH 7 and pH 8) tended to show a slight increase in cell degeneration at the end of the incubation period

On electrophoresis (Fig 3) the haemolytic and leukolytic activities were repeatedly found to have *identical* running characteristics

By the methods used (lysis of red cells and trypan blue uptake by thymocytes) the red cells were found to be more susceptible to damage than the thymocytes The ratio was at least 100 1, as judged by calculations based upon 50 per cent cell damage

DISCUSSION

The results show that mouse thymocytes are susceptible to lysis by haemolytic culture filtrates of *A calcoaceticus* The leukolytic and haemolytic activities showed many similarities The haemolysin (phospholipase C) has been shown to be markedly heat labile, to be activated by Mg^{++} (9) and to be inactivated by ethylendiaminetetraacetate (to be published)

The pH parameters of the leukolytic and haemolytic activities were also essentially the same, showing only slight differences between pH 7 and pH 8 and being lower at pH 6

The use of the vital dye technique counting as few as one hundred cells in each sample, cannot be expected to give accurate figures, even in repeated experiments Thus minor differences between pH 7 and pH 8 could not be evaluated by this method

The apparent difference in sensitivity of red cells and thymocytes might be due to differences between the test methods used (haemolysis and trypan blue uptake) However on the agar slides a marked zone of haemolysis occurred in 10–15 min whereas 60 min was necessary for leukolysis to become visible

Evidence that the haemolytic and leukolytic activities were due to the same enzymatic action was further supported by electrophoresis

Phospholipase C hydrolyzes various phospholipids into a phosphorus free lipid moiety and the corresponding monophosphate ester The enzyme would thus be expected to have profound effects on biological membranes containing one or more of the susceptible phospholipids Such activities have been described in for example clostridial α haemolysin (phospholipase C) (2 3 5 6 12) and in staphylococcal β haemolysin (sphingomyelinase C) (3 13 14)

Although the leukolytic activity of *A calcoaceticus* has been examined in a crude filtrate the striking similarities between the haemolytic and the leukolytic activities indicate that they may both be due to the action of the enzyme phospholipase C

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PROPERTIES OF PURIFIED PHOSPHOLIPASE C FROM *ACINETOBACTER CALCOACETICUS*

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Phospholipase C (phosphatidylcholine cholinephosphohydrolase EC 3 1 4 3) from *Acinetobacter calcoaceticus* was purified 20 fold by ultrafiltration and chromatography on DEAE-Sephadex The purified enzyme was markedly labile Mg^{2+} deoxycholate and albumin enhanced the enzymatic activity against lecithin while Ca^{2+}, Zn^{2+} and ethylendiaminetetra acetate inhibited the activity The temperature coefficient ($Q_{10°C}$) was 1 7 and optimum pH 8 2 8 6 The Michaelis constant was found to be lower than 250 µM At pH 8 3, lecithin phosphatidylethanolamine, phosphatidylserine and sphingomyelin were attacked, while at pH 6 3 only lecithin and sphingomyelin were susceptible to hydrolysis

The haemolytic and phospholipase C activities of *Acinetobacter calcoaceticus* have previously been shown to be closely associated (11, 12), and the production of phospholipase C in a chemically defined medium has been described (13) In the study reported here, a purified enzyme preparation was used for further investigation of phospholipase C activity

MATERIALS AND METHODS

Chemicals

Phospholipids were purchased from Koch Light Laboratories Colnbrook Bucks, England L α lecithin (PC), ex egg CHR, batch no 53399 phosphatidylethanolamine (PE), ex egg CHR, batch no 48360 L-3 phosphatidylethanolamine synthetic (PE syn) batch no 49731, phosphatidylserine (PS), ex bovine brain CHR, batch no 55495 and sphingomyeline (Sph) ex bovine brain CHR batch no 37795 A presumed molecular weight of 800 was used for determination of the molar concentrations of PC Bovine albumin (fraction V, from bovine plasma) was obtained from Armour Pharmaceutic Company, Eastbourne England, protamine (sulphate, 10 mg/ml) from Lovens Kemiske Fabrik, Copenhagen, Denmark and sodium deoxycholate from E Merck AG, Darmstadt, West Germany Casein was obtained locally All other chemicals were analytical grade

Bacterial Strain, Screening Methods and Cultivation Technique

The culture medium (13) the bacterial strain (1318/69) and the method for estimation of haemolytic activity (11) have been described earlier During purification protein was estimated as the absorbance at 280 nm in a Unicam S P 500 Spectrophotometer Low protein concentrations made specific assay methods unsuitable Purification and stability were examined by estimating the haemolytic activity The pH of the solutions used was measured at 25° C Bacteriolytic and proteolytic activities were investigated turbidimetrically as described by *Petit et al* (16) Proteolytic activity was examined with casein as

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substrate and bacteriolytic activity with *Streptococcus pyogenes* (group A), *Staphylococcus aureus* (strain Copenhagen) *Micrococcus lysodeicticus M roseus* and *Bacillus megaterium*

A slightly modified cultivation technique was used Erlenmeyer flasks each containing 1 l of medium were inoculated with 10 ml of a 24 h culture of the organism and incubated at 28° C the flasks being slowly agitated At late log phase (58 64 h) the cells were removed by centrifugation in a Servall Superspeed centrifuge (8 000 g 90 min 4° C) The supernatant was dialyzed for 24 h at 4° C against two successive portions of 20 volumes of 0 05 M tris pH 8 3 then re centrifuged and stored at —20° C until used Haemolytic activity ranged between 10 15 H U /ml

Ultrafiltration

The dialyzate was concentrated in an Amicon Ultrafiltration cell (Lexington Mass USA) at 4° C using a Diaflo membrane UM 20E at a N pressure of 3 kp/cm²

Column Chromatography

DEAE Sephadex A 50 (Pharmacia Uppsala Sweden) was equilibrated with 0 05 M tris pH 8 3 and filled into a column (2 5 × 36 cm) Concentrated dialyzate was applied to the column washed with 2–3 column volumes of buffer and then eluted by a linear NaCl gradient at 4° C (see Fig 1 for experimental details)

Polyacrylamide Electrophoresis

This was performed in a water cooled Page electrophoresis unit (Quickfit Instrumentation Stone Staffordshire England) at pH 9 5 according to *Hjertén et al* (8) Prior to electrophoresis the samples (0 3 ml) were dialyzed against the reservoir buffer diluted 1 5 in 3 per cent (w/v) sucrose Stacking was performed at 0 75 mA/tube (60 min) running at 2 5 mA/tube (90 min) followed by staining in 0 1 per cent w/v Amido Black Zones of haemolytic activity were detected by placing the discs in beds in a blood agar plate (37° C 30 min incubation)

Assays of Phospholipase C Activity

An Insonator model 500 Ultrasonic Disintegrator (20 kc/s 10 min) was used for the preparation of phospholipid sols Enzyme solutions (peak fractions from DEAE Sephadex chromatography) were mixed with phospholipid sol specific ions and detergent and incubated at 37° C unless otherwise specified Experimental details are described in the figures and tables Samples from the incubation mixtures were precipitated with 0 2 volumes of 30 per cent (w/v) trichloracetic acid (TCA) Sphingomyelinase C activity was estimated by the same method except that albumin was added as a coprecipitant according to *Maheswaran & Lindorfer* (15) Aliquots of the TCA supernatants (usually 0 1 ml) were used for determination of acid soluble phosphorus according to *Bartlett* (2) During the incubation the concentration of inorganic phosphorus did not increase and controls devoid of either enzyme or substrate showed constant and low phosphorus values

RESULTS

Purification

Fractionation with ammonium sulphate was not successful CM Sephadex and hydroxylapatite were unsuitable for chromatography Preparative gel filtration on Sephadex G 100 resulted in a considerable loss of activity

Chromatography on DEAE Sephadex showed a satisfactory elution pattern and a recovery of 70–100 per cent Column elution by a linear NaCl gradient was found to be superior to batch and step wise elution procedures A gradient elution experiment is presented in Fig 1 The peak fraction appeared at 0 1 M NaCl

On ultrafiltration undialyzed supernatant showed a greater loss of activity than dialyzate A procedure resulting in a 20 fold purification and a total recovery of 61 per cent is shown in Table 1 In the best of the peak fractions (fraction no 32), the purification was 27 3 The peak fractions (31 32 and 33) were concentrated to 1 ml, and portions of 0 3 ml were subjected to electrophoresis Crude dialyzate, concentrated in the same manner showed 10 protein bands whereas no protein bands were visible in the purified material In both a single haemolytic zone appeared 9 mm from the starting point

Stability

In contrast to the stability of crude dialyzate, purified material was markedly labile Peak fractions from DEAE Sephadex chromatography showed an 85 per cent loss of haemolytic activity at 4° C and a 40 per cent loss at —20° C when stored for 10 days

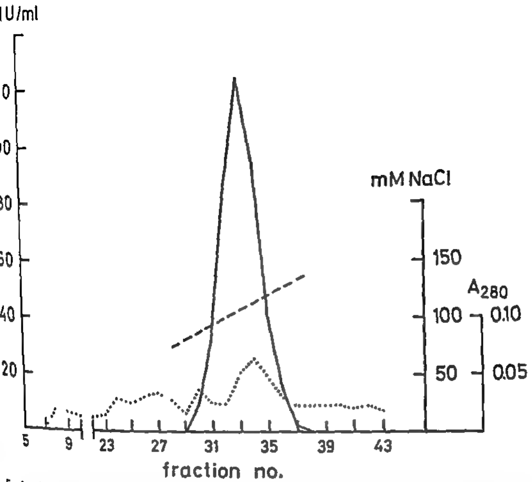

Fig 1 Chromatography of Phospholipase C on DEAE-Sephadex 55 ml ultrafiltrate (98 5 H U /ml, A_{280} 0 430) in 0 05 M tris pH 8 3 were applied to a DEAE Sephadex column, 2 5 × 36 cm, equilibrated with the same buffer After washing with 250 ml buffer, elution was carried out by a linear NaCl-gradient (- - -) (final concentration 0 4 M NaCl, total volume 600 ml, in 0 05 M tris pH 8 3) Fraction volumes were 10 ml flow rate 0 3 ml/min All operations were performed at 4° C Haemolytic activity (——) was assayed according to the standard method (11) One haemolytic unit (H U) was defined as the amount of haemolysin that would produce 50 per cent haemolysis of 1 ml 1 per cent human red cell suspension ( ) Absorbance at 280 nm

E 1 Purification of Phospholipase C by Ultrafiltration and DEAE Sephadex Chromatography

	Volume ml	Purification	Spec act Haem act /A_{280}	Recovery %
le dialyzate	500	1	125	100
afiltrate	55	1 8	229	81
ractions DEAE S	80			61
ed, peak fractions	30	20 5*	2560	38

In the best peak fraction, the purification was 27 3 (specific activity 3410) Experimental details described in the text and in Fig 1

Cysteine, dithiotreitol, mercaphoethanol (1 mM), glycine (10 mM), glycerol (200 mg/ml), or ethanol (5 per cent (v/v)) did not increase the stability Albumin (1 mg/ml) increased the activity by more than 100 per cent, but did not change the slope of the inactivation curve Crude supernatant was not inhibited by 5 mM 5,5 dithiobis (2 nitro benzoic acid) (DTNB) Urea (8 mM) had an inhibitory effect on both crude dialyzate and purified material The activity was reduced by 40 per cent by oxygen bubbling of crude dialyzate for 1 h, and was not restored by cysteine or mercaptoethanol (5 mM)

Properties

The experiments were performed with enzyme solutions purified according to the procedure presented in Table 1 Unless specified, PC was used as a substrate

Heating prior to incubation resulted in complete inhibition of phospholipase C activity in 15 min at 45° C and in 5 min at 56° C Albumin (1 mg/ml) and $MgCl_2$ (5 mM) did not increase heat stability

Table 2 shows that Mg^{++} and deoxycholate enhanced the activity which is in accord with previous observations (13) Deoxy[...]late and albumin increased the activity [...] or without Mg^{++}, but did not potentiate [...] other Protamine showed a moderate st[...]latory effect, independent of Mg^{++} [...] Ca^{++} and ethylendiaminetetraacetate (ED[...] disodium salt) inhibited the activity, [...]plete inhibition occurring with 4 mM ED[...] in the absence of Mg^{++} Optimal conce[...]tions of Mg^{++} and deoxycholate were 5 [...] and 1 mg/ml, respectively (Fig 2) T[...] concentrations were used in subsequent [...]periments Higher concentrations decre[...] the activity

At 24° C, the reaction rate was con[...] for 90 min, while at 37° C a slight devi[...] occurred between 60 and 90 min (Fig [...] The temperature quotient ($Q_{10°C}$), c[...]lated from the rectilinear parts of the gra[...] was 1 7

Optimum pH was 8 2–8 6 (Fig 4) F[...] shows that the initial reaction rate was p[...]tically unaffected by a decrease in the [...]centration of PC from 6 25 mM to 0 25 [...] At 0 25 mM PC, the substrate was comp[...]ly hydrolyzed in 18 min At 4 mM and [...] mM PC the reaction rate remained cons[...]

TABLE 2 *Effects of Various Compounds on Phospholipase C Activity*

	Activity %
Control (DEAE Sephadex eluate + lecithin sol + water)	100
5 mM $MgCl_2$	224
5 mM MgCl + 4 mM Na EDTA	196
5 mM MgCl + 0 1 mM Zn acetate	108
5 mM MgCl + 1 mg/ml Albumin	328
5 mM MgCl + 1 mg/ml Na deoxycholate	387
5 mM MgCl + 1 mg/ml Na-deoxycholate + 1 mg/ml Albumin	390
5 mM $MgCl_2$ + 1 mg/ml Protamine	164
1 mg/ml PC Protamine	161
1 mg/ml Albumin	132
1 mg/ml Na deoxycholate	206
5 mM $CaCl_2$	23
4 mM Na EDTA	0
0 1 mM Zn acetate	28

2 5 ml DEAE Sephadex eluate 0 9 ml lecithin sol (30 mM suspended in 0 05 M tris pH 8 3) aqueous solutions containing the various ionic compounds (final volume 4 0 ml final concentrat[...] as in table) were mixed and incubated at 37° C for 1 h Acid soluble phosphorus (phosphorylc[...] liberated from lecithin) was estimated according to Bartlett (2)

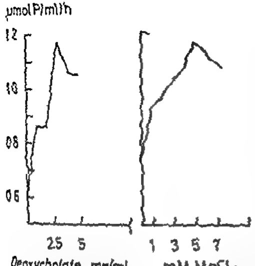

Fig 2 Effect of $MgCl_2$ and Sodium Deoxycholate on Phospholipase C Activity 1 20 ml DEAE-Sephadex eluate 0 45 ml 30 mM lecithin sol and aqueous solutions of MgCl and deoxycholate were mixed to a final volume of 2 0 ml Final concentration of $MgCl_2$ in the deoxycholate series was 5 mM and of deoxycholate in the MgCl series 3 mg/ml Incubation lasted for 1 h at 37° C Increase in acid soluble phosphorus was estimated according to Bartlett (2)

for 27 min resulting in 38 per cent hydrolysis According to Fig 5 the Michaelis constant (K_m) was found to be lower than 0 25 mM To determine K_m (on the basis of the initial reaction rate) it would be necessary to estimate very small differences in phosphorus at considerably shorter time intervals than used in this experiment Thus, for technical reasons an accurate determination of K_m was omitted

The calibration graph in Fig. 6 shows linearity within the range of enzyme activities used maximum velocity 2 2 µmol P ml/h)

Table 3 shows that at pH 6 3 only PC and Sph were attacked, while at pH 8 3 all the phospholipids tested were susceptible to hydrolysis PC showing the highest values

Proteolytic or bacteriolytic activities were not detected

DISCUSSION

Purification was hampered by the lability of the enzyme To obtain a sufficiently active enzyme preparation a short lasting and simple purification procedure had to be used

The stability with regard to DTNB suggests that reactive SH groups were not essential to the enzymatic activity This was supported by the failure to reactivate oxygen treated enzyme or to increase its stability by SH reagents

The marked heat sensitivity of this enzyme is shared by, for example, *Pseudomonas fluorescens* phospholipase C (6), but contrasts with the thermostability of phospholipase C from *Bacillus cereus* (9) and from *Clostridium perfringens* (14) The mechanisms involved have not been systematically investigated The absence of measurable amounts of protease and bacteriolytic enzymes seems to preclude the enzyme having

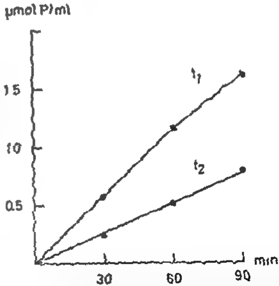

Fig 3 Effect of Temperature on Phospholipase C Activity The incubation mixture incubation and estimation of enzyme activity were the same as in Fig 2 Final concentrations were 5 mM $MgCl_2$ and 3 mg/ml deoxycholate t_1 37° C, t_2 24° C Temperature coefficient

$$Q_{10°C} \; \frac{K\ value_{t_1} \times 10}{K\ value_{t_2} \times (t_1\ t_2)} = 1\ 7$$

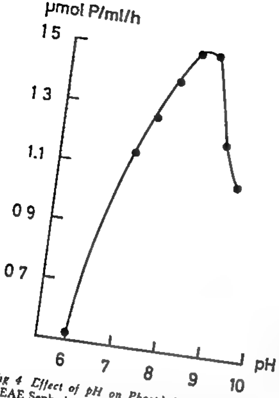

Fig 4 Effect of pH on Phospholipase C Activity DEAE Sephadex eluate was dialyzed overnight at 4° C against 2 × 100 volumes of distilled water 3 parts dialyzate were mixed with 1 part buffer at the respective pH s The buffers were pH 6 0 and pH 7 0 0 4 M tris maleate pH 7 4 9 2 0 32 M tris with 0 5 M NaCl To 0 8 ml enzyme/buffer solution was added 0 3 ml 30 mM lecithin sol dissolved in 145 mM NaCl and $MgCl_2$ and deoxycholate to a final volume of 1 33 ml Final concentrations were 5 mM $MgCl_2$ and 3 mg/ml deoxycholate Incubation lasted for 1 h at 37° C Assay for enzyme activity was the same as in Fig 2

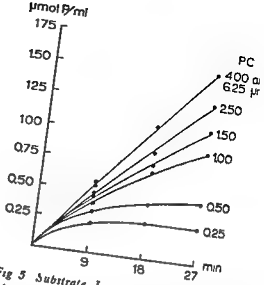

Fig 5 Substrate Variation Kinetics of Phospholipase C DEAE Sephadex eluate was concentrated four fold by ultrafiltration To 0 8 ml concentrate was added lecithin sol $MgCl_2$ and deoxycholate as described in Fig 4, except that the concentration of lecithin was varied (final concentrations are seen in the figure), and samples were collected at 0, 9, 18 and 27 min

been broken down by other enzymes Repeated freezing and thawing of concentrated crude dialyzate tended to inactivate the enzyme and to split the haemolytic activity into two electrophoretically distinct fractions (unpublished observations) Although conclusive evidence is not at hand inactivation by polymer formation may be one of the features of the enzyme

The stimulatory effect of Mg^{2+} has previously been demonstrated in crude enzyme preparations, employing haemolysis (11), egg yolk turbidimetry (12) and splitting of pure lecithin (13) as test methods Inhibition by

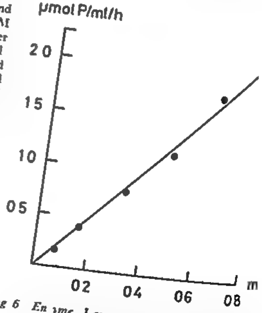

Fig 6 Enzyme Variation Kinetics of Phospholipase C DEAE Sephadex eluate was concentrated two-fold by ultrafiltration Portions of 0 08, 0 16, 0 32, 0 48 0 64 and 0 80 ml were diluted in the filtrate fluid to portions of 0 80 m These portions were incubated with lecithin so $MgCl_2$ and deoxycholate and examined on enzymatic ac described in Fig 4

TABLE 3 *Effect of Phospholipase C on Various Phospholipids*

Phospholipid	Activity % pH 6 3	pH 8 3
Phosphatidylcholine (PC)	100	100
Phosphatidylethanolamine (PE)	0	30
Phosphatidylethanolamine synthetic	0	31
Phosphatidylserine (PS)	0	64
Sphingomyelin (Sph)	96	77

Phospholipid sols (24 mg/ml suspended in 145 mM NaCl) were prepared as described in the text. Portions of 0 3 ml were added to DEAE Sephadex eluate, $MgCl_2$ and deoxycholate as described in Fig 4 At pH 6 3, the eluate was used after being dialyzed overnight at 4° C against 200 volumes of 0 1 M tris-maleate pH 6 3 Incubation and assays of enzymatic activity were performed as described in Fig 4 The activity was estimated as the percentage of the activity with respect to PC at the respective pH s

EDTA also indicates that divalent metal ions are essential to the enzymatic activity The effect of metal ions on phospholipase C from other sources is heterogeneous Phospholipase C from *Staphylococcus aureus* (β haemolysin, sphingomyelinase C) was also activated by Mg^{++} and inactivated by EDTA (15) In contrast to this phospholipase C from *Cl perfringens* (1, 14) and *Ps fluorescens* (6) were activated primarily by Ca^{++}, while rat liver phospholipase C (sphingomyelinase C) (7) and *B cereus* phospholipase C (9) were Ca^{2+} independent Zn^{+}, which inhibited the enzyme in question, has also been shown to inactivate phospholipase C derived from a marine plankton alga (3), while, for example *Cl perfringens* phospholipase C was activated by Zn^{++} (17)

All these findings seem to support the view of *Diner* (5), working with the clostridial enzyme, that in at least some of the phospholipase C enzymes, there is a direct participation of a metal ion in the enzymatic mechanism in addition to an action of the metal ion on the micellar phospholipid substrate (1)

Deoxycholate has been extensively used as a detergent for the preparation of soluble phospholipid substrates The inhibitory effect of higher concentrations of deoxycholate and Mg^{++} may be due to the formation of insoluble Mg deoxycholate

The stimulatory effect of albumin has not been further investigated, and although it has been used for this purpose by others (5, 17), its mechanism of action is uncertain

The finding of a $Q_{10°C}$ of 1 7 is in good agreement with the results of previous investigations (haemolysis 1 8 (11), egg yolk turbidimetry, 1 9 (12))

With PC as a substrate, optimum pH (8 2–8 6) also corresponds to the values found in egg yolk turbidimetry (pH 8 0–8 4) and in the haemolysis reaction, where the activity increased slightly from pH 7 6 to pH 8 0 Optimum pH was higher than in clostridial phospholipase C (pH 7 0–7 6 (14)), in phospholipase C from *Pseudomonas aeruginosa* (pH 7 0–7 5) (10)) and in other phospholipases

Determination of K_m was dependent upon the physical state of the incubation mixture, containing the substrate in a micellar form An accurate determination of K_m would have been of greater significance if it could have been performed in a completely water soluble mixture

Diner (5), employing H_3 labelled dioleyl lecithin and *Neurospora* lecithin as substrates, estimated K_m of clostridial phospholipase C at 70 μM and 40 μM, respectively, and discussed the problems of K_m determination in media containing PC in a micellar phase

Phospholipase C from *A calcoaceticus* revealed a broad substrate specificty The methods used do not however permit to rule out the possibility that more than one phospholipase was present in the purified material

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THE FORMATION OF EXTRACELLULAR PROTEOLYTIC ENZYMES BY *STAPHYLOCOCCUS AUREUS*

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Three different proteolytic enzymes could be identified in supernatants from cultures of *Staphylococcus aureus*, strain V8, if grown in a casein hydrolysate yeast extract medium (CCY_I) One protease was not produced if the yeast extract was omitted from the medium This protease was inactivated by ethylene diamino tetra acetic acid (EDTA, 5 0 mM at 20° C for 30 min) If calcium ions (final conc 2 5 mM) were added to the yeast extract free medium, the EDTA sensitive protease was formed in amounts approaching those obtained in the complete medium The formation of the EDTA stable enzymes was not affected by calcium ions The two EDTA stable activities could be separated by isoelectric focusing one with a pI of 4 0 and the other with a pI of 9 4 The formation of the EDTA-stable protease was stimulated by yeast extract

Most micrococci have been shown to produce extracellular proteolytic enzymes These may serve a nutritional role by cleaving proteins to peptides and amino acids which can be transported into the bacteria It has been shown earlier that complex media containing casein hydrolysate and yeast extract gave high yields of extracellular proteolytic enzymes from *Staphylococcus aureus*, strain V8 (2) Defined media, according to *Gladstone* (10) and *Mah et al* (20), gave low bacterial yields of this strain and no proteolytic activity could be detected in the culture supernatants In order to study the regulation of protease formation in staphylococci it was thus of importance to analyse which medium components were essential for the appearance of extracellular proteolytic activity

MATERIALS AND METHODS

Bacterial strain Staphylococcus aureus, strain V8, obtained from Prof *G P Gladstone*, University of Oxford England, was used in this study The stock culture was transferred monthly on agar slants

Culture media A modification (CCY_I) of the CCY med [illegible]

[illegible], 40 g, yeast extract (Difco) 10 g sodium β glycerophosphate 20 [illegible] sodium lactate (50 per cent), 10 ml, $Na_2HPO_4 \cdot 2H_2O$, 1 g, KH_2PO_4 0 4 g, $(NH_4)_2SO_4$ 1 g DL-tryptophane 80 mg L-cystine, 100 mg thiamine, [illegible] mg nicotinic acid 4 mg, $MgSO_4 \cdot 7H_2O$ 20 mg, $MnSO_4 \cdot 4H_2O$ 10 mg, $FeSO_4 \cdot 7H_2O$ 6 mg, citric acid 6 mg distilled water to a final volume of 1000 ml The vitamins were heat sterilized separately at pH 4 5 in a 100 fold concentrated solution The trace metal

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were also sterilized separately in a 100 fold concentrated solution. The bulk of the medium was sterilized at 120° C. Polypropylene glycol P 2000 (Dow Chemical Co., Midland, Mich.) was added (0.05 ml/l) as an antifoam agent before sterilization.

Inocula. Precultures were grown for 18 h in Trypticase Soy Broth (BBL, Cockeysville, Md., U.S.A.). The bacteria were collected by centrifugation; they were resuspended in the fresh medium and used as an inoculum. The initial density of bacteria in the culture was 0.2 mg/ml (dry weight).

Cultivation techniques. The bacteria were cultivated in a stirred fermentor (Biotec FL 103, Biotec, Stockholm, Sweden) with a working volume of 2.5 l and in 1 l baffled Erlenmeyer flasks with a culture volume of 100 ml on a rotary shaker (120 r.p.m., 50 mm displacement). The air flow through the stirred fermentor was 1 l per min and the impeller speed was 650 r.p.m. All cultivation experiments were run at 37° C. In the stirred fermentor pH was kept constant at 7.2 by the aid of a titrator unit (Radiometer TTT1, Copenhagen, Denmark). Bacterial growth was assessed by dry weight measurement.

Assay for proteolytic activity. Proteolytic activity was determined according to *Kunitz* (16) using heat denatured casein at pH 7.5 as the substrate. Minor modifications of the assay procedure has been described earlier (2). The extent of proteolysis was determined by reading the absorbancy of perchloric acid soluble degradation products at 280 mμ in a Zeiss spectrophotometer model PMQ II. One unit was defined as an increase by 1.0 in the absorbance at 280 mμ in 30 min at 37° C.

Determination of intracellular and cell bound proteolytic activity. Cells harvested during exponential growth were collected by centrifugation and washed once in 0.1 M Tris HCl buffer at pH 7.0. The cells were suspended in 0.05 M Tris HCl buffer at pH 7.5 containing 10^{-3} M $CaCl_2$ and lysostaphin (10 μg/ml, Mead Johnson, Evansville, Ind., U.S.A.). The density of the suspended cells was 5 mg/ml (d.w.) which was the same as that in the culture. The suspension was kept at 22° C for 2 h. Proteolytic activity was determined in the supernatant and in the resuspended pellet after centrifugation at 6000 × g for 20 min.

Metal analysis. The Mg^{++} and Ca^{++} content of the complete medium, yeast extract, and the Mg^{++} content of samples of spent medium was determined with the aid of an atomic absorption spectrophotometer (EEL model 140).

Isoelectric focusing. Isoelectric focusing was performed as described by *Vesterberg et al.* (33). Fifty ml samples were dialysed against glycine (0.5 per cent w/v) and applied to a 110 ml column (LKB-Produkter, Stockholm-Bromma, Sweden). Carrier ampholytes pI 3.0–10.0 (LKB Produkter) were used to establish the pH gradient. The focusing experiments were run for 48 h at 4° C with a potential of 500–600 V. The contents of the column were collected in 3 ml fractions and assayed for proteolytic activity after pH determination of each fraction.

RESULTS

Accumulation of proteolytic activity during growth. The increase of extracellular proteolytic activity during growth in the stirred fermentor of *Staphylococcus aureus*, V8 in the casein hydrolysate yeast extract medium CCY_1 is shown in Fig. 1. The bacterial growth started after a lag of less than 30 min. Protease formation displayed a lag of about 2.5 h. The increase in proteolytic activity occurred from 2.5 to 4.5 h of cultivation during which time bacterial density increased from 2.5 up to 6.5 mg/ml. After the cessation of protease formation the bacterial growth continued, but at a slower rate for several hours, and a final cell density of 10 mg/ml was achieved after 10–12 h. After the proteolytic activity had reached its maximum it was inactivated to about 40 per cent during the continued cultivation (Fig. 1). The fact that the total proteolytic activity was not inactivated in 24 h although the initial rate of inactivation was high indicated the presence of at least two different proteases.

The true extracellular nature of the proteolytic activity was shown by the absence of any intracellular pool. Only trace amounts of proteolytic activity were found in the cytoplasmic fraction of bacteria taken from the culture after four hours of cultivation. The proteolytic activity of the cell debris fraction was about 10 per cent of the total activity in the culture. The bacteria were lysed by lysostaphin (10 μg/ml). Treatment for 2 hours resulted in 90 per cent lysis of the cells.

The influence of the medium components on the formation of proteolytic enzymes was also studied. Omission of yeast extract from the nutrient medium resulted in a marked decrease in the bacterial growth. After 7 h of growth the dry weight of cells was 2 mg/ml, and prolonged incubation only resulted in an increase up to 3 mg/ml at 24 h

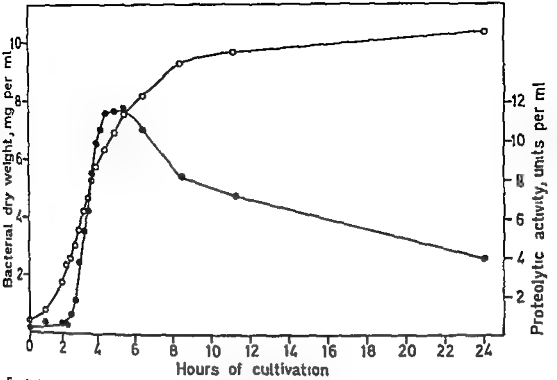

Fig 1 Bacterial growth and extracellular proteolytic activity in a culture grown in the CCY$_I$ medium
O—O bacterial dry weight mg per ml ●—● proteolytic activity, units per ml.

The protease formation was also low (1 5 units/ml) I seemed probable that the growth limiting factor was magnesium ions, regarding the high Mg^{++} content of yeast ex tract as compared to casein hydrolysate (4) Analysis of the complete medium and sam ples taken from shake flask cultures with increasing amounts of Mg^{++} showed that Mg was the growth limiting factor in the yeast extract free medium The final cell density obtained in yeast extract free medium with different concentrations of Mg is shown in Fig 2 In the complete CCY$_I$ medium Mg was not the growth limiting factor The me dium without yeast extract supplemented with Mg at a final concentration of 0 7 mM was used for further experiments This me dium will be referred to as the CC medium

The growth and protease formation in the CC medium in the stirred fermentor are shown in Fig 3 The final yield of protease was only 15 per cent of that obtained in the CCY$_I$ medium although the bacterial dens ty was only reduced by 30 per cent Obviously, some factor other than magnesium in the yeast extract stimulated the protease forma tion

The fact that several bacterial proteases,

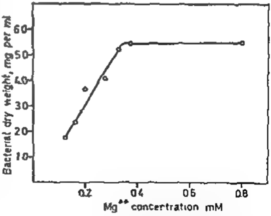

Fig 2 Influence of magnesium ions on the final yield of bacte ia The experiments were performed in shake f ask us ng CCY$_I$ medium without yeast extract to wh ch was added $MgSO_4$ 7H O

before and after the EDTA treatment and the activity of each portion was plotted against the time of cultivation (Fig 5) The EDTA sensitive and the EDTA stable part of the proteolytic activity appeared at different times during the cultivation thus supporting the assumption that at least two different proteases had been demonstrated Treatment by EDTA at a concentration of 5 0 mM for 30 min at 20° C was henceforth used as the standard procedure for the determination of the EDTA stable and the EDTA sensitive proteases in different samples

The proteolytic activity produced in the CC medium (*i e* calcium deficient) was not inactivated by EDTA (Fig 6) while 50 per cent of the proteolytic activity, produced in CC medium to which calcium had been added to a concentration of 2 5 mM was inactivated (Fig 7) The amounts of EDTA-stable proteolytic activity formed were not affected by the addition of Ca^{++} to the me-

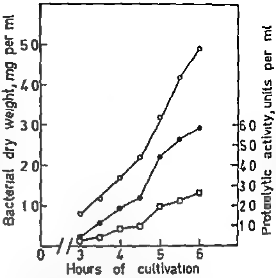

Fig 7 Extracellular proteolytic activity in a culture grown in the CC medium to which calcium ions to a final concentration of 2 5 mM had been added The proteolytic activity was determined before and after the treatment of the samples by EDTA (5 0 mM at 20° C for 30 min) ○—○ bacterial dry weight mg per ml ●—● proteolytic activity before EDTA treatment units per ml □—□ proteolytic activity after EDTA treatment units per ml

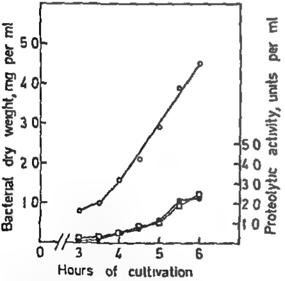

Fig 6 Extracellular proteolytic activity in a culture grown in the calcium deficient CC medium The proteolytic activity was determined before and after treatment of the samples by EDTA (5 0 mM at 20° C for 30 min) ○—○ bacterial dry weight mg per ml ●—● proteolytic activity before EDTA treatment units per ml □—□ proteolytic activity after EDTA treatment units per ml

dium (comp Fig ▮ and 7) Obviously the presence of yeast extract or Ca^{++} in the culture medium stimulated the formation of an EDTA sensitive protease which was not produced in the absence of these compounds Addition of Ca^{++} to supernatants from cultures grown in the calcium deficient CC medium did not increase the proteolytic activity No reactivation of EDTA inactivated protease was obtained by incubation with Ca^{++} at a concentration of 10 mM for several hours or by *dialysis against 10 mM* Ca^{++} for 24 hours

Comparison of the final yields of protease activity (calculated per mg of bacterial dry weight) in the CCY_I medium the CC medium and the calcium supplemented CC medium showed that yeast extract stimulated the formation of the EDTA stable proteolytic activity in addition to its effect on the EDTA sensitive protease (Table 2) The amounts of EDTA sensitive protease were always higher in the CCY_I medium than *in* the calcium supplemented CC medium The figures given

TABLE 2 *The Maximum Amounts of EDTA Sensitive and EDTA-Stable Proteolytic Activity per mg of Bacterial Dry Weight in Different Media The Proteolytic Activity was Determined before and after Treatment of the Samples by 5 0 mM EDTA for 30 Min at 20° C*

Culture medium	EDTA sensitive proteolytic activity units per mg of bacteria	EDTA stable proteolytic activity, units per mg of bacteria
CCY_I	1.6 ± 0.4	1.5 ± 0.2
CC medium	<0 1	0.7 ± 0.2
CC medium +2 5 mM Ca^{2+}	0.7 ± 0.2	0.7 ± 0.2

in Table 2 are the results of ten experiments in each medium run on different occasions The divergence between values from different experiments could be attributed to variations in the aeration efficiency

Separation of EDTA-stable proteolytic activities Culture supernatants containing both EDTA-sensitive and EDTA stable proteolytic activities were subjected to isoelectric focusing in order to separate the two proteases (Fig 8) Two peaks of proteolytic activity were found with pI = 4 0 and pI = 9 4 The total recovery was 10–15 per cent in several experiments Fifty per cent of the inactivation occurred during dialysis against 0 5 per cent glycin prior to the isoelectric focusing The proteolytic activity of the two peaks was not inactivated by EDTA Evidently the EDTA sensitive protease was inactivated during dialysis and the iso electric focusing and the EDTA stable proteolytic activity consisted of two components with different isoelectric points Samples taken from cultures grown in the CC-medium which contained no EDTA-sensitive proteolytic activity were also subjected to isoelectric focusing and

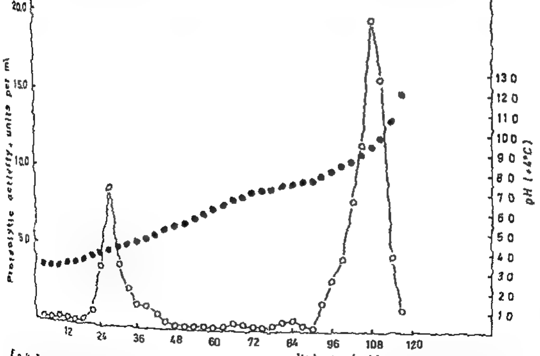

Fig 8 Isoelectric focusing of crude culture supernatant from *Staphylococcus aureus* strain V8 grown in the CCY_I medium The experiment was performed in a 110 ml column for 48 h at 400 600 V Three ml fractions were collected ●—● pH of each fraction measured at 4°C ○—○ proteolytic activity

yielded the same two peaks at pH 4.0 and pH 9.4. The total recovery was about 60 per cent, indicating that not only the EDTA sensitive protease was inactivated by this method. Attempts to find a third component after iso-electric focusing (i.e. the EDTA sensitive protease) by removing the carrier ampholytes and the sucrose from all the fractions collected after the focusing experiment and by adding Ca^{++} were unsuccessful.

DISCUSSION

The appearance of the proteolytic activity in the culture fluid during active growth and the partial inactivation during the stationary phase indicates that it is a true extracellular activity (25). This was supported by the fact that very little cell bound or intracellular activity could be demonstrated. The proteolytic activity in the culture supernatant seems to be a mixture of at least three different enzymes, one of which could be inactivated by EDTA. Two EDTA stabile enzymes were separated by iso-electric focusing. *Birch-Hirschfeld* (3) purified the gelatinolytic activity from *Staphylococcus aureus* and it appeared as one enzyme active against gelatine and casein. *Elek* (8) however suggested that several proteinases must be produced by staphylococci. *Robinson et al.* (26) could demonstrate two distinct proteolytic enzymes during the purification of α-haemolysin by chromatography on carboxymethylcellulose. *Tirunarayanan & Lundblad* (32) also demonstrated two different protease from *Staphylococcus aureus* by the aid of electrophoresis. *Sandvik & Fossum* (29) showed that coagulase positive strains of micrococci fell in the same group according to classification with antiproteinase serum. They could only find one antiprotease activity in the γ-globuline fraction. After separation by paper electrophoresis of crude supernatants from *Staphylococcus aureus* only one protease could be found (28).

The amounts of calcium ions which had to be added to the medium to obtain maximum stimulation of the protease formation greatly exceed the amounts present in the yeast extract. Since the casein hydrolysate contains high amounts of phosphate, calcium will precipitate as calcium phosphate. The calcium present in yeast extract might be chelated and thereby prevented from precipitation but still able to maintain its protease stimulating function. It has earlier been showed by *Hames* (13) that Mg^{++} and Ca^{++} stimulated growth and protease formation of several bacterial species in a synthetic medium. Magnesium stimulated growth without stimulating protease formation and calcium stimulated protease formation without affecting growth. *Shooter & Wyatt* (30) showed that magnesium and calcium were essential as components of the nutrient medium for some strains of *Staphylococcus aureus*. They suggested that the growth stimulating effect of calcium was due to the requirement of calcium for the formation of protease.

Studies of the mechanism by which calcium stimulates the production of EDTA sensitive protease are now in progress. The fact that no reactivation of EDTA inactivated protease and no activation of an inactive enzyme which might have been secreted by bacteria grown in calcium deficient media was obtained supported the assumption that the function of calcium ions was to stabilize the labile protease. The role of calcium in the formation of an extracellular protease by a *Sarcina* strain (Coccus P) was shown by *Bissell et al.* (4). This protease was only found when calcium ions were present in the culture medium and in the absence of calcium the excreted protease was rapidly autodigested. An extracellular proteinase from *Micrococcus caseinolyticus* purified by *Desmanseaud & Hermier* (7) was 100 per cent inactivated by EDTA and was partially reactivated by Ca^{++}. Calcium ions were also shown to prevent autodigestion of this protease. Similar effects of calcium ions on proteases from different *Pseudomonas* species have been described by *Morihara* (22, 23, 24). The mechanism of the inducing effect of calcium on protease formation in Ba-

cillus megaterium shown by *Gorini* (12) and *Fayyaz Ud Din et al* (9) is not known

The production of the EDTA stable pro teolytic activity was totally repressed during the first three hours of cultivation (Fig 3) Also, the formation of EDTA sensitive protease was repressed (although not completely) during the first 3 or 4 hours of cultivation Many extracellular protease formed by various microorganisms are known to be repressed by carbohydrates in the culture medium (6, 14, 17, 18, 31) and also by amino acids (6, 14, 19, 21, 27, 31) According to these reports the protease production should be repressed until the concentration of repressor has decreased to a critical level Until the different proteolytic enzymes have been purified and a method for their individual estimation has been developed no meaningful study of the regulation of their synthesis can be performed Furthermore, the accumulation of these enzymes in the extracellular fluid also involves the transport and, possibly, activation, which might be regulated by specific mechanisms

Other environmental conditions may also influence the formation or release of extracellular enzymes Staphylokinase which is also produced by strain V8 is formed mainly under oxygen limited growth (1) The onset of protease accumulation in the stirred fermentor experiments corresponds well to the time at which dissolved oxygen tension of the culture approaches zero (unpublished data) After dissolved oxygen has reached zero, bacterial growth continues at a linear rate proportional to the oxygen transfer rate (15) The linear growth period can be seen in Fig 1 to range between a bacterial density of 2.5 and 6 0 mg per ml

The addition of yeast extract to the casein hydrolysate medium (CC-medium) doubled the yield of EDTA-stable proteolytic activity (Table 2) Preliminary experiments showed that the protease stimulating factor had a molecular weight between 1000 and 5000, probably a peptide acting as an inducer Since the proportion of the two EDTA stable proteases can not yet be determined the rate of induction could not be calculated It seems, however, to be of low order Proteases from *Aeromonas proteolytica* (17, 18), *Arthrobacter* (14) and *Vibrio parahaemolyticus* (31) were induced by mixtures of peptides The rates of induction were rather low which might be explained by the fact that these proteases were also repressed by amino acids

Before a more detailed study of the regulation of protease formation by *Staphylococcus aureus*, strain V8, can be done, the three proteases must be purified and characterized and a method must be found by which to estimate these individually in mixed samples

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HAEMOPOIETIC DEFECTS IN MICE INFECTED WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS

I. *The Enhanced X-Ray Sensitivity of Virus Infected Mice*

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Seemingly paradoxical observations were made with mice exposed to infections with the LCM virus and treatment with X rays. The X irradiation could afford a beneficial effect upon the course of the viral infection or cause a serious aggravation. The beneficial effect was associated with X ray treatment given prior to the inoculation of virus. This pre irradiation effectively prevented an antiviral immune response and protected mice against lethal intracerebral challenges with the virus. However, doses of X rays which were sublethal for normal mice provoked a lethal disease when given to mice previously inoculated with the virus. Fatal outcomes were observed with persistent, tolerant virus carriers and with i. p. infected adult mice, that is in cases of normally inapparent LCM virus infections. The X ray induced deaths were apparently not related to any enhanced growth of the virus in the irradiated mice. Clinical findings and reconstitution experiments strongly indicated that the striking increase in the sensitivity to X rays was caused by a haemopoietic defect in the LCM virus infected mice. The nature of this haemopoietic disorder is not readily explained. Thus several observations made it unlikely that the LCM virus had any direct damaging effect upon the haemopoietic cells. Moreover, the findings with the persistent tolerant virus carriers provided evidence against any immunopathological basis of the disorder.

From observations of the symptomless persistent virus carrier state which follows congenital or *neonatal* infection of mice with lymphocytic choriomeningitis (LCM) virus (9, 16) and from studies of infected cells in tissue cultures (4, 7), it appears that the LCM virus generally has no direct cytopathic effect. On the *other* hand convincing evidence of a immunopathological basis of the acute LCM disease in mice has come from the dramatic effect of immunosuppressive treatment on the course of the infection. Since *Rowe's* original observations (10), several investigators (4, 5, 6) have demonstrated that X-irradiation may prevent deaths and pathological lesions caused by LCM and, in addition, it has been shown that neonatal thymectomy, ALS and a number of immunosuppressive drugs may offer a varying degree of protection against the LCM virus (11, 3, 6). However, with other neurotropic viruses the pathogenesis appears to be quite different in that damage is caused directly by a harmful viral effect and the diseases are aggravated by immunosuppressive treatments (8).

During our work with LCM virus it was

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noticed that the effect of X-ray treatment of LCM infected animals was rather complex. In contrast to the protective effect which has been described we found that irradiation under certain circumstances provoked a very serious aggravation. From our experimental results it appeared that the LCM virus might cause a profound dysfunction of the haemopoietic system, resulting in enhanced sensitivity to X rays. It is the purpose of this and the following paper (1) to present our data concerning this virus-induced haemopoietic defect and to discuss the implications of the findings.

MATERIALS AND METHODS

Virus. One strain of LCM virus was used exclusively. It was obtained from Dr *Traub* (Tubingen, Germany). The virus preparations employed were ten per cent suspensions of spleens from infected mice. The virus titrations were carried out by intracerebral inoculation (0.03 ml) of serial ten fold dilutions into groups of ordinary 12–14 g white Swiss mice. Titration end points were calculated according to the Karber method and expressed as LD_{50}/0.03 ml.

Mice. Except for those used for the titration purposes, all the mice employed belonged to a strain of highly inbred C_3H mice. Persistent tolerant virus carriers (virus carriers) were produced by inoculating newborn mice within the first 18 hours of life with 1000 LD_{50} of the virus. As previously described (18) this treatment results in a state of immunological tolerance and the mice will carry virus in high titres in blood and organs throughout life. Acute infections of mature mice were produced by intraperitoneal or intracerebral injections of 1000 LD_{50} of virus. When given by the intraperitoneal route this dose of virus was not fatal but caused a symptomless transient viraemia with maximal virus titres about the sixth day. When the virus was given intracerebrally, however, the infection was usually fatal but, contrary to what is observed in Swiss mice, a few C_3H mice occasionally survived. All mice immune to the LCM virus were mothers of infected babies. They develop a symptomless infection and when the babies are taken from them at the age of 4 weeks they are highly immune (15). All the mice used, except the mothers, were aged 2–4 months.

Preparation of cells. Cell suspensions from 1) livers of near term foetuses, 2) spleens and lymph nodes of immune mice and 3) femoral bone marrows were prepared in Hank's balanced salt solution and counted as previously described (15).

Fluorescent antibody staining. Films of suspended bone marrow cells were dried on cover slips and fixed in acetone for 10 min at −20° C. The cells were stained for 30 min at 37° C with the fluorescein isothiocyanate conjugated globulin fraction of hyperimmune mouse serum (kindly supplied by *Rode Pedersen*).

Histology. Smears of femoral bone marrows were stained with May Grunwald Giemsa and sections of the sternum were stained with haematoxylin eosin after fixation in Bouin's solution.

Complement fixation test. Antigen was prepared from spleens of infected mice. The standard procedures used have been described in a previous paper (17). The method was altered only in that Sever's microtechnique (12) was employed.

X irradiation. Irradiation was administered by a Siemens Stabilipan theraphy machine operated at 200 kV and 16 mA with 0.9 mm Cu and 0.5 mm Al filtration. The recipients were radially arranged on a rotating platform during the irradiation. Blood samples were drawn in parallel from all groups of mice involved in the same irradiation experiment.

RESULTS

The effect of X-irradiation on viraemic mice. In several experiments with LCM virus carriers we observed that the lethal dose of X-rays was considerably lower for these mice than for normal controls. Although the virus carriers show no overt clinical signs it was conceivable that the enhanced sensitivity was associated with some disorder characteristic of the persistent carrier state. For comparison we therefore included acutely infected mice in our studies. In the first experiment these mice were infected by the intraperitoneal route and irradiated six days later, that is at the stage of maximum viraemia.

Fig 1 shows the results of the administration of various doses of X-rays to groups of virus carriers, acutely infected mice and normal controls. A group of ten acutely infected mice received no irradiation and remained perfectly healthy during the period of observation. As seen from the figure the survival rates of irradiated virus infected animals were highly abnormal. It is evident that both the highest mortality and the shortest survival time were associated with the acute type of infection. Thus the results di

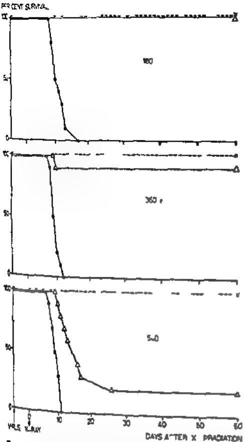

Fig 1 Mortality of virus carriers, acutely infected mice and normal controls after receiving 180, 360 or 540 r. Δ——Δ virus carriers, O——O acutely infected mice inoculated i p with 10^3 LD_{50} of virus six days prior to X-irradiation, x– – –x normal mice. Ten mice per group

not support the suggestion that the enhanced sensitivity to X rays might be connected with some disorder peculiar to the persistent tolerant carrier state

The effect of pre-irradiation Seemingly our findings above formed a sharp contrast to the protective effect of X-irradiation on the course of LCM described previously. This phenomenon was therefore studied with the mouse and virus strains used in our experiments. The protective effect has been shown by the administration of X rays prior to intracerebral inoculation of LCM virus. In the following experiment irradiation was therefore given one day before the inoculation of virus, and both intraperitoneally and intracerebrally infected mice were employed (see Fig 2). In contrast to our findings above all the irradiated, i p infected mice remained healthy, and the observations on the i c inoculated animals clearly confirmed the beneficial effect of pre-irradiation on this normally lethal type of infection

X-irradiation and viraemia One might suspect that the increased mortality observed in our first experiment (Fig 1) could be related to an abnormal, enhanced growth of virus provoked by the X-ray treatment. The influence of X-irradiation on the viraemia of virus carriers and acutely infected mice was therefore examined. A group of 17 virus carriers was given 400 r of X rays. Individual blood virus titres were determined prior to irradiation, four days later and in the ten surviving mice ten days after the irradiation. The mean virus titres in each of these three titration experiments were $10^{3.4}$, $10^{3.1}$ and $10^{3.8}$ LD_{50}/0.03 ml respectively. That is, no evidence of enhancement of virus multiplication was found in the irradiated virus carriers

Table 1 shows the virus titres in irradiated and untreated, acutely infected mice. It appears

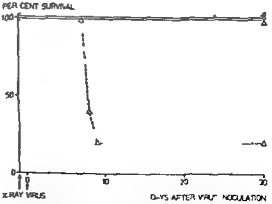

Fig 2 The effect of X-irradiation given one day prior to i p or i c inoculations of LCM virus. Dose of X rays 270 r. Dose of virus 10^3 LD_{50}.
O——O pre-irradiated i p inoculated mice,
■ – – O unirradiated i p inoculated mice,
Δ——Δ pre-irradiated i c inoculated mice,
Δ– – Δ unirradiated i c inoculated mice.
Ten mice per group

TABLE 1 *The Effect of X-irradiation on Virus and CF Titres of Acutely Infected Mice*

Groups*	Day of X-irradiation§ relative to virus inoculation†	Mean blood virus titres			Mean CF titres 16 days after virus inocul
		6 days after virus inocul	10 days after virus inocul	16 days after virus inocul	
Post-irradiated mice	+6	$\leq 10^{1.3}$	$10^{.8}$	10^{1} "	16"
Pre-irradiated mice	−1	10	$10^{3.4}$	$10^{1.7}$	<8
Unirradiated mice		$10^{1.5}$	$\leq 10^{1.1}$	$\leq 10^{0.7}$	$10^{?}$

* Ten mice per group § X-ray dose 360 r † Inoculated i p with 10^{3} LD_{50} of virus
" Mean value from only 2 survivors

that the irradiation of acutely infected mice six days after virus inoculation resulted in some increase of the viraemia. However, as can be seen, the growth of virus in animals exposed to the harmless pre-irradiation treatment was even higher. As a whole the results were not suggestive of a direct relation between the X-ray induced aggravation and any enhancement of virus multiplication.

Determination of CF antibody titres indicated that no humoral immune response was elicited by the irradiation of virus carriers and, in the case of acutely infected mice, that the X-ray treatment given prior to virus inoculation was effectively immunosuppressive.

Bone marrow studies. The deaths among irradiated LCM infected mice were not associated with convulsions or other signs of cerebral lesions; however, we observed a progressive loss of weight, severe anaemia and widespread haemorrhages in many tissues. The picture suggested a fatal haemopoietic disease, i e an abnormal sensitivity of the haemopoietic system to normally sublethal doses of X-rays. In the search for some virus-induced defect in the haemopoiesis we studied the bone marrows of infected non-irradiated animals. Microscopy, cell counts and fluorescent antibody staining of femoral marrows were performed.

The microscopy of the bone marrows from the virus carriers did not reveal any pathological changes, and the total cell counts of suspensions from the femurs were similar to values obtained with normal controls. However, when mice infected six days previously were examined, it was found that the marrows were depleted of many of the more mature erythroid and granuloid cells, and the relative numbers of primitive blast cells were greatly increased. Moreover, the cell counts from the femoral shafts were decreased by about fifty per cent. The change which had occurred in the marrow of the acutely infected mice could also readily be seen by gross examination. It appeared bloodless and pale yellow.

The proportion of virus-infected cells in the bone marrows was examined by fluorescent antibody staining as described under methods. In the virus carriers we found between one and three per cent positive cells. Acutely infected mice were studied 3, 6, 9 and 12 days after i p inoculation of virus. Only in the second group did we find very small numbers of positive cells in the course of screening of several thousand cells, i e less than 0.1 per cent. During acute infections the viraemia is maximal about the sixth day. As shown in Table 1, the titres amounted to about $10^{?}$ LD_{50}/0.03 ml at this time. With virus carriers, however, 100-fold higher values were recorded. The results therefore suggested that there is a simple correlation between the numbers of virus-infected cells in the bone marrow and the virus titres in the blood.

Reconstitution of irradiated mice. To obtain further information about any defect in the haemopoietic system of LCM infected mice we attempted to reconstitute the an-

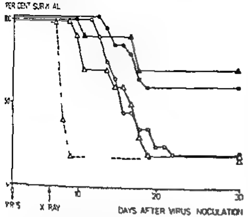

Fig 3 The effect of reconstitution with foetal liver cells on the mortality of irradiated, acutely infected mice 270 r of X rays were given six days after i p or i c inoculations with 10^3 LD_{50} of virus Injections of foetal liver cells were given i v within 3 hours after the irradiation ●——● i p infected irradiated mice transplanted with foetal liver cells (10×10^6 or 20×10^6 cells per mouse), ○——○ i p infected irradiated mice, ▲——▲ i c infected, irradiated mice transplanted with foetal liver cells (20×10^6 per mouse), △——△ i c infected irradiated mice, △- - -△ i c infected unirradiated mice Twenty mice per group of i p infected animals Ten mice per group of i c infected animals

mals with normal haemopoietic tissue after X irradiation Adult mice infected either i p or i c six days previously, and virus carriers were given doses of X rays, which were sublethal for normal mice Within three hours after the irradiation one half of each group was injected intravenously with ten to twenty million foetal liver cells (Fig 3 and 4)

From Fig 3 it can be seen that only four out of 20 i p infected mice survived after irradiation without transplantation, whereas 12 mice were saved in the corresponding transplanted group A similar effect was observed with i c infected mice in that two out of ten survived the irradiation when untreated, whereas seven transplanted mice survived The enhancement of the survival rate of exogenously reconstituted mice was significant at the one per cent level ($X^2 = 9\ 85$)

It is apparent that the survival time of i c inoculated mice treated with X-rays on day six was significantly increased, as compared with the corresponding non irradiated animals The median survival time (of mice dying during the observation period) was 7 7 days in the non-irradiated group and 15 5 days in the irradiated group Furthermore, it is seen that the survival times of irradiated i c and i p infected mice were almost identical (median survival time 15 5 and 15 3 days respectively)

The protective effect of the injection of foetal liver cells was even more pronounced in the irradiated virus carriers As can be seen from Fig 4 the transplantation treatment was found to be 100 per cent curative It is perhaps conceivable that this result was caused by some adoptive immunization effect associated with the transplantation Individual blood virus titres of transplanted and non transplanted mice were therefore determined 20 days after the X irradiation The results did not give the slightest evidence of any difference between the two groups of mice The titres were very similar to those observed with untreated virus carriers Furthermore, all sera were negative when tested for complement fixing antibodies

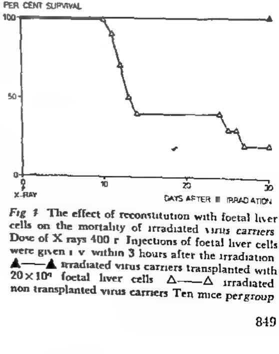

Fig 4 The effect of reconstitution with foetal liver cells on the mortality of irradiated virus carriers Dose of X rays 400 r Injections of foetal liver cells were given i v within 3 hours after the irradiation ▲——▲ irradiated virus carriers transplanted with 20×10^6 foetal liver cells △——△ irradiated non transplanted virus carriers Ten mice per group

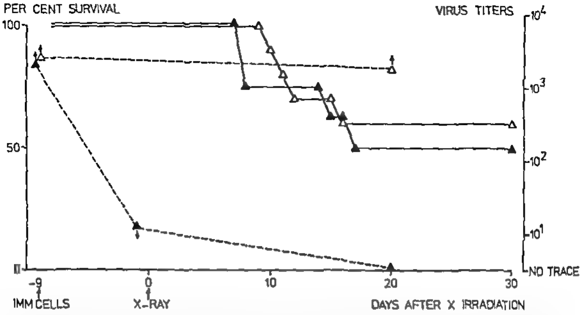

Fig 5 The effect of adoptive immunization of virus carriers on their sensitivity to X irradiation 100×10^6 syngeneic immune spleen and lymph node cells were injected i p nine days prior to X irradiation Dose of X rays 400 r ▲——▲ survival of adoptively immunized virus carriers (eight mice) △——△ survival of non transplanted virus carriers (ten mice) ▲ – ▲ mean blood virus titres of adoptively immunized virus carriers (arrow indicates that titration end points were not reached for all mice in a group) △ – △ mean blood virus titres of non transplanted virus carriers

The experiments above strongly indicate that the abnormal sensitivity of LCM infected mice to X rays is related to some defect in their haemopoietic system

X irradiation of adoptively immunized virus carriers It seemed somewhat surprising that the haemopoietic reconstitution of irradiated transplanted virus carriers could proceed so effectively in the presence of high persistent titres of virus This finding suggested that the haemopoietic dysfunction was not directly associated with the actual viraemia To test this assumption virus carriers were injected with 100×10 pooled syngeneic immune spleen and lymph node cells prior to X irradiation This adoptive immunization is known to bring about a rapid elimination of virus (17) It is seen from Fig 5 that the elimination was almost complete eight days after the transplantation and furthermore that the irradiation given on day nine did not produce any relapse of the viraemia It is however apparent that the adoptively immunized mice showed no increased survival rate as compared with untreated controls The same observation was made with virus carriers irradiated 30 days after the transplantation i e at a time when no trace of virus was left in any mouse The findings thus constituted further evidence against any simple relationship between the haemopoietic dysfunction and viraemia

DISCUSSION

Large sublethal doses of X rays given during the week before antigenic stimulation are known to be highly immunosuppressive (13) Moreover, in the present experiments an effective abrogation of the antiviral response in pre irradiated mice was indicated by the enhanced growth of virus and the absence of any antibody production (Table 1) The protective effect of pre irradiation on the course of LCM is therefore readily explained on the basis of an injurious role of the immune response in the pathogenesis Direct evidence of a harmful role of the immune

response against LCM virus has recently been reported by *Gilden et al* (2) These authors showed that immune spleen cells, when injected into immunosuppressed viraemic mice, caused typical fatal lesions in the central nervous system

The grave disease provoked by the irradiation of virus carriers or adult mice infected six days previously showed no clinical similarity to the classical fatal LCM virus infection The clinical findings, the bone marrow studies and the reconstitution with foetal liver cells strongly indicated that this disease was a consequence of some virus induced defect in the haemopoietic system However, the basic nature of this haemopoietic disorder is not readily explained

Several findings made it rather unlikely that the LCM virus had any direct damaging effect on the haemopoietic cells Thus the haemopoietic regeneration of pre irradiated animals and the reconstitution of irradiated virus carriers transplanted with foetal liver cells seemed to proceed in a normal way in the presence of high titres of virus And conversely the abnormal sensitivity to X rays persisted in the virus carriers despite an effective termination of the viraemia by adoptive immunization Furthermore, the proportion of virus infected cells in the bone marrow was very low as revealed by the fluorescent antibody staining and least in the most sensitive acutely infected mice

Considering the prominent role of the immune response in the pathogenesis of LCM and the highly different effects on the immune response of irradiation given before and after antigenic stimulation one might suspect that immune mechanisms were involved in the fatal haemopoietic disease of mice infected prior to irradiation It is well known that the administration of X rays during the days after a primary stimulation is not associated with any decrease of the antibody response although the peak titres may be somewhat delayed (14) Thus one might postulate some immune mediated cytotoxic effect on the haemopoietic cells in these mice infected before the irradiation This suggestion however offers no ready explanation of the findings in the tolerant virus carriers and, furthermore it is quite inconsistent with observations presented in the following paper (1)

It has been reported earlier (4) that X rays failed to cure intracerebral LCM infection when the inoculation of virus preceded the irradiation In accordance with our findings, the treatment only led to prolongation of the survival time However, from the present data it appeared that the irradiation might, in fact, afford a protective effect upon the intracerebral disease although it remained obscure because of the fatal haemopoietic disease Evidence for this suggestion came from the beneficial effect of injected foetal liver cells and from the almost identical survival times of irradiated i c and i p infected mice (Fig 3)

The authors are indebted to Mag *J Ambrosen* Radiofysisk Laboratorium Finsen Institute for providing X irradiation facilities and wish to express their gratitude to Mrs *I Lystlund Nielsen* and Mrs *B Højer Simonsen* for skillful technical assistance

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HAEMOPOIETIC DEFECTS IN MICE INFECTED WITH LYMPHOCYTIC CHORIOMENINGITIS VIRUS

2 The Viral Effect upon the Function of Colony-forming Stem Cells

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In the preceding report (3) experiments were described which suggested the occurrence of haemopoietic disorders in mice infected with the LCM virus. Immunological disorders associated with the LCM virus infections of mice are well known It was considered that both haemopoietic and immunological defects might be caused by some virus induced interference with the stem cells in the infected mice Attempts were therefore made to estimate the numbers and the function of stem cells in mice infected with the LCM virus Haemopoietic colony assays were employed and both acutely infected mice and persistent, tolerant virus carriers were examined The findings suggested a decrease in colony forming stem cells in the bone marrow during the acute infection However, the most striking observation in the acutely infected mice was of a profound inhibition of the colony forming response of stem cells in these animals The influences exerted upon the stem cell response seemed to be quite specific, in that the response of immunocompetent cells was almost unimpaired when examined at the same time The inhibition was not caused by a direct viral effect upon the stem cells nor by any immune mediated damage Experiments with the adult, persistent, tolerant virus carriers indicated that the haemopoietic colony formation was not inhibited in these mice despite a pronounced viraemia However, their numbers of colony forming stem cells amounted to about fifty per cent of the normal values and seemed to agree with their relative increase in sensitivity to X rays It is suggested that virus induced interference with the function of haemopoietic stem cells plays a central role for the tolerogen effects of the LCM virus

From the preceding report (3) it appeared that lymphocytic choriomeningitis (LCM) virus might cause a profound dysfunction of the haemopoietic system in mice In accordance with the non cytopathogenic nature of LCM virus, the results were not consistent with any direct damaging effect of the virus on the haemopoietic cells and, furthermore, the findings with persistent, tolerant virus carriers (virus carriers) did not support any immunopathological basis of the disorder

Another obscure, but highly distinctive, feature of the LCM virus is its ability to induce immunological tolerance when injected into immature mice (17) This viral effect upon the immune system accounts for profound differences between neonatal and adult LCM virus infections (6)

Experiments by *Wu et al* (19) and *Tren-*

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lin et al (16) have brought forward very strong arguments for a derivation of the mature immunocompetent cells from the haemopoietic stem cells. It seems, therefore, reasonable to suspect an intimate relationship between the haemopoietic and the immunological influences of the LCM virus. A viral effect upon the stem cells might be very relevant to both phenomena.

To obtain further information about the haemopoietic disorder, and to throw light upon a possible connection between the haemopoietic and immunological defects, we studied the stem cells in LCM virus infected mice, employing the spleen colony method of *Till and McCulloch* (14). It is the purpose of this report to present the data from these experiments and to discuss the implications of the results.

MATERIALS AND METHODS

The virus, the virus titrations, the mice and the preparation of cellular suspensions have been described in the preceding report (3). The normal C_3H mice, the virus carriers and the acutely infected mice employed for the experiments were all aged 2–3 months, while the immune mothers were about 4 months old. The virus carriers were produced by inoculating newborn mice within the first 18 hours of life with 10^3 LD_{50} of the virus. The acute infections of mature mice were produced by intraperitoneal injections of 10^4 or 10^5 LD_{50} of virus. From our experiments it appeared that both doses of virus caused the same profound dysfunction of the haemopoietic system, however, when given intraperitoneally to normal mice they gave rise to no apparent symptoms.

Haemopoietic colony assays. The method of irradiation has been described previously (3). For the study of exogeneous colony formation recipient mice were given 800 r and within three hours after the irradiation they were injected intravenously with pooled bone marrow cells. Five recipients were used per pool, and a minimum of five donors were employed for the preparation of any pool. In all transplantation assays the donors and recipients were of the same sex. Endogeneous colonies were studied in mice which received 495, 585 or 675 r.

Six days after irradiation the mice were killed and their spleens and sternums fixed in Bouin's solution. The spleens were examined by a histological study of a longitudinal mid section through the hilus. A single midline section has been shown to provide an adequate sample for estimating the colonies in a given mouse (5). Spleen colonies were scored using criteria similar to those employed by *Curry and Trentin* (4). The sternums were studied microscopically on longitudinal sections through the mid zone of the bone. In each of these sections several bone lined marrow zones could be examined.

Assay for plaque-forming cells. Spleens were prepared and assayed individually for counting of direct plaque-forming cells. The Jerne haemolytic plaque method was employed, as modified for use with glass microscope slides (11). Agarose (L'Industrie Biologique Française S. A.), 0.5 per cent in Eagle's minimal essential medium with Hank's salts, 0.4 ml, sheep erythrocytes, 10 per cent, 0.05 ml, and the spleen cell suspensions, 0.1 ml, were mixed and poured onto microscope slides previously coated with 0.1 per cent agarose in water. The slides were incubated for one hour at 37° C in a humid atmosphere containing 5 per cent CO_2. Guinea pig serum, diluted 1:10 in Eagle's medium, was added and incubation was continued for 2 hours. The plaques were counted with indirect illumination using a magnifying glass. The numbers of plaque forming cells in the cellular suspensions were calculated from the counts of duplicate slides.

RESULTS

The colony-forming ability of bone marrow cells from virus infected donors in normal recipients. Morphological studies of the bone marrows from infected animals (3) were not very informative as regards the nature of the haemopoietic defects. An investigation of the function of the bone marrow cells was therefore carried out employing the spleen colony method of *Till & McCulloch* (14). Comparisons were made between the colony-forming abilities of bone marrow cells from 1) normal mice, 2) virus carriers and 3) acutely infected mice injected i.p. with virus six days previously. In the first series of experiments (Table 1) irradiated normal mice were used as recipients of the bone marrow cells.

It can be seen from the table that 0.5×10^6 normal bone marrow cells gave rise to about 15 colonies per mid section of the recipient spleens. However, somewhat unexpectedly, the normal looking bone marrow cells from *the virus carriers did not* produce any colonies. Even when doses as high as 5×10^6 cells

TABLE 1 *Spleen Colonies in 800 r Irradiated Normal and Immune Mice Injected with Various Preparations of Bone Marrow Cells*

Marrow donors	Recipients	No of cells injected ($\times 10^6$)	Addition of virus*	Mean no of colonies per spleen section ± SD	
				Experiment 1	Experiment 2
Normal	Normal	0 1	—	2 8±1 6	
Normal	Normal	0 5	—	16 4±2 5	13 8±4 3
Virus carriers	Normal	0 5	—	0	0
Virus carriers	Normal	5 0	—		0
Acutely infected mice§	Normal	0 5	—	0	
Acutely infected mice§	Normal	5 0	—	0	0
Normal	Normal	0 5	+	0	0
Normal	Normal	5 0	+		0 6
Normal	Immune	0 5	+		19 4±0 9

* 10^3 LD_{50} of virus per 0 5 ml were added to bone marrow cell suspensions and incubation carried out for 30 min at room temperature prior to injection

§ Inoculated i p with 10 LD_{50} of virus six days previously

were employed not a single colony was found in any of the recipients The histological picture showed an empty splenic stroma similar to that observed with irradiated non-transplanted mice As regards the acutely infected mice, the results revealed that their bone marrow cells were just as ineffective as those from the virus carriers

During the transplantations of bone marrow cells from virus carriers and from acutely infected mice, a simultaneous transfer of infectious virus was unavoidable To explore any possible effect of this virus on the colony forming abilities of normal stem cells the following experiments were included To the suspensions of normal bone marrow cells were added 10^3 LD_{50} of LCM virus per 0 5 ml i e per one transplantation dose and about 30 min later the cell virus mixture was injected into irradiated mice As may be seen from Table 1, the addition of virus caused a striking inhibition of the colony formation in normal recipients The results emphasized a prompt effect of the virus on the function of the haemopoietic stem cells Moreover, this viral effect might be the explanation of the failure of the growth of stem cells from the infected donors Haemopoietic stem cells might, therefore, be present in these mice in spite of the negative transplantation results

The colony forming ability of bone marrow cells from virus infected donors in immune recipients To the experiments mentioned above was added a control group of recipients which were immune to the LCM virus After irradiation this group received part of the mixture of virus and normal bone marrow cells used in experiment 2 recorded in Table 1 As is apparent from the last figures in the table, the numbers of colonies observed in these mice were at normal levels It appeared from these results that the stem cells were not damaged by the *in vitro* incubation with the virus and moreover, that some immune mediated inhibition of the growth of the virus in the recipients was decisive for the colony formation

As a consequence of these findings, irradiated immune recipients were employed in a further study of the colony forming stem cells in the virus infected animals Table 2 shows the results from two experiments in which bone marrow cells from virus carriers and from mice infected six days previously were injected into groups of irradiated immune mothers The findings indicated that the bone marrows of the infected mice in fact contained a certain number of haemopoietic stem cells and that the function of these cells was unimpaired in mice capable of

TABLE 2 *Spleen Colonies in 800 r Irradiated Immune Mice Injected with Bone Marrow Cells from Normal or LCM Virus Infected Mice*

Marrow donors	Recipients	No of cells injected ($\times 10^6$)	Mean no of colonies per spleen section ± SD Experiment 1	Experiment 2
Normal	Immune	0.25	8.0±4.5	7.6±4.2
Virus carriers	Immune	0.25	6.2±2.8	2.8±2.5
Acutely infected mice*	Immune	0.25	4.2±2.4	5.0±1.4

* Inoculated i p with 10^{3} I D_{50} of virus six days previously

TABLE 3 *Spleen Colonies in 800 r Irradiated Virus Carriers Injected with Bone Marrow Cells from Normal Mice or Virus Carriers*

Marrow donors	Recipients	No of cells injected ($\times 10^6$)	Mean no of colonies per spleen section ± SD Experiment 1	Experiment 2	Experiment 3	Experiment 4
Normal	Virus carriers	0.25	9.3±1.5	10.5±1.3		
Normal	Virus carriers	0.50			≥16.3*	17.3±2.6
Virus carriers	Virus carriers	0.25	5.5±1.9	3.0±2.6		
Virus carriers	Virus carriers	0.50			7.8±1.3	10.3±2.8

* The large numbers and sizes of colonies made counting difficult and in the case of one spleen impossible

eliminating the virus As can be seen the numbers of colonies obtained with the cells from infected donors were somewhat smaller than the numbers produced by normal bone marrow cells A decrease in colony forming stem cells in the bone marrows of virus carriers was confirmed by observations reported below The relative proportions of colonies of erythroid granulocytic and undifferentiated morphology did not indicate any apparent differences between the various kinds of donor cells

The colony-forming ability of bone marrow cells in virus infected recipients In the experiments above the stem cells of the virus infected mice were enumerated by means of their ability to form colonies in immune recipients Additional information was however needed in order to assess the function of the haemopoietic system in the virus carriers and in the acutely infected mice In the following experiments virus carriers and acutely infected mice were therefore, used as recipients In the first series of experiments irradiated virus carriers were examined after injections of stem cells derived from either normal mice or from virus carriers In the second series irradiated acutely infected mice were studied after injections of stem cells from normal mice or from acutely infected donors

As can be seen from Table 3 the stem cells from both normal mice and virus carriers were able to proliferate and form colonies in virus carriers that is, in the presence of high titres of infectious virus in the blood and organs As observed in the experiments described above the numbers of colonies produced by the bone marrow cells from virus carriers were smaller than those obtained with normal cells To evaluate a difference between the two kinds of cells it seemed justifiable to pool the data from the six independent experiments recorded in the Tables 2 and 3 With normal bone marrow cells 8.0, 7.6, 9.3, 10.5, 8.2 and 8.7 colonies

TABLE 4 *Spleen Colonies in 800 r Irradiated Acutely Infected Mice* Injected with Bone Marrow Cells from Normal Mice or Acutely Infected Mice*

Marrow donors	Recipients	No of cells injected ($\times 10$)	Mean no of colonies per spleen section $\pm$ SD	
			Experiment 1	Experiment 2
Normal	Acutely infected mice*	0 5	3 0 ± 1 0	6 2 ± 4 1
Normal	Acutely infected mice*	5 0		§
Acutely infected mice†	Acutely infected mice*	0 5	0 4	0 2
Acutely infected mice†	Acutely infected mice*	5 0		1 0

* Inoculated i.p with 10 LD$_{50}$ of virus six days prior to irradiation
§ The large numbers of colonies made counting impossible
† Inoculated i p with 10 LD$_{50}$ of virus six days previously

were found per $0\ 25\times10^6$ injected cells, and with virus carrier cells the corresponding figures were 6 2, 2 8, 5 5, 3 0, 3 9 and 5 2 colonies per $0\ 25\times10$ injected cells From these data means and standard deviations were calculated $8\ 7\pm1\ 1$ colonies per $0\ 25\times10^6$ pooled normal bone marrow cells and $4\ 4\pm1\ 4$ colonies per $0\ 25\times10$ pooled virus carrier cells The difference between the numbers of colony forming stem cells in the two kinds of cell populations was significant at the 0 001 level

It was a characteristic feature that the spleen colonies in the virus carriers were larger than those observed in other recipients Their increased size was readily seen both by gross examination and by microscopy

As regards the recipients inoculated with virus six days prior to the irradiation, it was found that they permitted the growth of stem cells from normal mice (Table 4) The numbers of colonies were somewhat below the normal values, but formed a sharp contrast to the negative findings with recipients inoculated with virus on the day of the irradiation (Table 1) The ability of bone marrow cells from acutely infected mice to form colonies in the recipients infected six days previously, however, seemed to be very poor Even a large dose of 5×10 bone marrow cells produced rarely distinct colonies and caused only some scattered granulocytic repopulation of the spleens

Reconstitution of the sternal bone marrow In addition to the spleens, the sternal bone marrows of the recipient mice were also examined Marrows of irradiated (800 r) non-transplanted mice were found to be depleted of virtually all haemopoietic cells, and the reconstitution in uninfected recipients of normal cells was readily recognized as small discrete foci of undifferentiated or granulocytic cells The examination of the mice employed for all the experiments above showed that the repopulation of the sternal bone marrows was very closely correlated with the colony formation in the spleens The findings indicated that the stem cells lodged in the marrows and the spleens were influenced by the LCM virus in exactly the same way Corresponding to the large size of the spleen colonies an extensive proliferation was observed in the marrows of virus carriers In contrast to the smaller foci in normal mice, many of the bone lined marrow zones of the virus carriers were almost completely occupied by crowded haemopoietic cells

Endogeneous colony formation The haemopoietic stem cell activity in virus infected mice was also studied by means of the endogeneous spleen colony assay Groups of virus carriers acutely infected mice inoculated with virus six days previously and normal controls were exposed to various doses of X-rays, and six days later their spleens were prepared for histological examination The mean endogeneous colony counts from this experiment are plotted in Fig 1 As can be

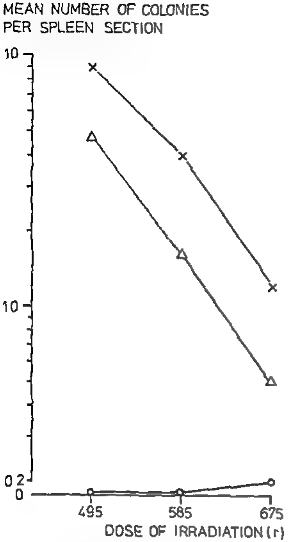

Fig 1 Mean numbers of endogenous spleen colonies in mice given varying doses of X rays six days previously O——O acutely infected mice inoculated i p with 10^3 LD$_{50}$ of virus six days prior to X irradiation Δ—— Δ virus carriers x——x normal mice The mean colony numbers of virus carriers and normal mice given 585 r were calculated from the colony counts of 12 and 16 mice respectively The remaining points are the mean colony counts of 5–6 mice

seen colony formation was almost completely inhibited in acutely infected mice at all doses of irradiation, and these findings corresponded to the negative results obtained with the transplantations of bone marrow cells between acutely infected mice (Table 4) The virus carriers and the normal mice however supported the growth of endogeneous stem cells and showed a dose dependent reduction of the colony counts similar to that reported by others (15) It appeared that the virus carriers were inferior to the normal mice as regards the numbers of colonies produced From the colony data of mice given 585 r a significant decrease of endogeneous colony formation ($p < 0.01$) was calculated by means of the Wilcoxen rank test The mean counts in virus carriers and normal mice were 1.6 and 4.0 respectively These figures closely agreed with the evaluation made above from the results of the transplantation assays and suggested a reduction of the stem cell pool in adult virus carriers to about half the normal level The endogeneous colony data of virus carriers, as well as those of acutely infected mice, seemed to be directly relevant to the enhanced X ray sensitivities recorded in the previous paper (3)

The proliferative responses of stem cells and of immunocompetent cells in the spleens of acutely infected mice An important question as to the nature of the inhibition of colony formation in acutely infected mice was that of the specificity of the effect Did the virus only interfere with the proliferation and differentiation of stem cells or did it affect other kinds of cells in a similar way? To answer this question an attempt was made to relate the viral effect on the stem cells to the effect on the functions of immunocompetent cells The colony forming response of stem cells and the immune response against sheep erythrocytes have features in common in that differentiation and extensive rapid cellular proliferation are involved in both processes furthermore, these responses may be assessed in the same organ namely the spleen

Fig 2 illustrates the proliferative responses of stem cells in acutely infected mice and normal controls The results are based on total cell counts of individual spleens from 40 mice The experiment was carried out in the following way Forty normal mice were given 800 r of X rays The mice were then devided into four groups which received dif

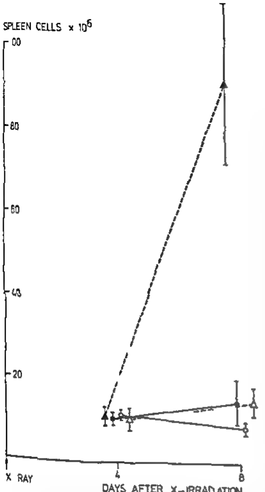

Fig 2 The proliferative responses of haemopoietic stem cells in the spleens of irradiated mice Forty normal mice were given 800 r of X rays and then divided into groups of ten mice Group 1 (●——●) received normal bone marrow cells and virus Group 2 (▲ – –▲) received normal bone marrow cells Group 3 (○——○) received virus Group 4 (△– – –△) was left untreated 0.5×10 bone marrow cells and/or 10^3 LD_{50} of virus were used per recipient and injections were made i v and/or i p respectively within three hours after the irradiation Four and eight days later five mice were killed per group and total spleen cell counts were made. The mean cell counts and standard deviations are shown in the figure

ferent treatments within 3 hours after the irradiation Ten mice were injected i v with 0.5×10 normal bone marrow cells and i p with 10^3 LD_{50} of LCM virus Ten mice were injected with the cells only and ten mice with the virus only A fourth group was left untreated after the irradiation Four and eight days later five animals from each group were killed and their spleen cells counted It can be seen from Fig 2 that neither transplantation nor virus infection influenced the cell counts in the course of the first four days During the next period of four days a striking increase in cell numbers occurred in the transplanted uninfected recipients The counts of transplanted, acutely infected mice however, were almost unchanged and similar to the values obtained from the non transplanted groups As might have been expected from the spleen colony data above the results indicated an almost complete inhibition of the proliferative response of stem cells in irradiated acutely infected mice

To expose any possible effect of the virus upon the cells involved in the immune response, the appearance of haemolytic plaque forming cells in the spleens of acutely infected mice injected with sheep red blood cells were examined The numbers of plaque forming cells were determined four days after intravenous injections with 2×10^8 erythrocytes that is, corresponding to the peak response of normal mice As can be seen from Table 5 more than 10 antibody forming cells appeared in the spleens of infected mice when antigen was given either simultaneous with or four days after the inoculation of virus The numbers obtained were comparable to those recorded with normal mice As a contrast to the severe inhibition of colony forming stem cells the results indicated an almost normal course of the processes involved in the immune response during the first eight days of the virus infection

DISCUSSION

The findings presented in this report have brought forward additional and convincing evidence for several of the conclusions reached in the foregoing paper (3) The occurrence of haemopoietic disorders in mice infected with LCM viru was confirmed by the observations of a profound viral influence on

TABLE 5 *Haemolytic Plaque Forming Cell Responses in Spleens of Normal and Acutely Infected* Mice*

	Day of virus inocul relative to PFC assay	Mean no of PFC per spleen§ ± SD	
		Non primed	Primed†
Normal mice		145± 46	195000±48000
Acutely infected mice	−4	347±113	143000±55000
Acutely infected mice	−8	474±149	129000±55000

* Inoculated i p with 10^3 LD_{50} of virus
§ Five mice per group
† Injected i v with 2×10^8 SRBC four days prior to PFC assay

the function of the colony-forming stem cells As to the nature of the haemopoietic failure, the spleen colony data from the immune recipients of bone marrow cells from infected animals emphasized that haemopoietic stem cells were neither killed nor irreversibly damaged by the virus (Table 2) Moreover, from the observations on the colony formation in virus carriers (Table 3) containing high titres of infectious virus it seemed very unlikely that the virus had any direct influence on the stem cell function Furthermore it was observed (see Table 1) that colony formation was inhibited in recipients given 800 r of X-rays prior to the injections of virus and bone marrow cells that is, in animals which were not capable of an appreciable immune response against virus antigen Immune mediated damage, which plays a prominent role in the pathogenesis of acute LCM, would not therefore, appear to be involved either

Several experiments indicated that the haemopoietic reconstitution was most seriously influenced in the acutely infected mice These animals were in some way rendered incapable of supporting the proliferation and/or differentiation of haemopoietic stem cells Somewhat surprising crucial events seemed already to occur during the first few days after virus inoculation, that is prior to any pronounced viraemia In fact some kind of normalization was observed as early as six days after the inoculation At this time exogeneous, normal stem cells were operating at least to some extent as shown by their curative (3) and colony forming (Table 4) abilities The experiments did not, however, suggest any corresponding release of the function of endogeneous stem cells in the acutely infected mice

The mechanism by which the LCM virus exerted its influence on the stem cells in the acutely infected animals is not clear It is well established that a series of both humoral factors and microenvironmental influences provide stimuli for the normal differentiation and proliferation of haemopoietic stem cells (10 Interference with one or more of these regulating processes in the course of acute LCM virus infections might afford a natural explanation of the observed effect It has recently been suggested that interferon may act as an inhibitor of haemopoietic colonies grown *in vitro* (9) A mechanism involving interferon is, however, not likely to account for the present findings, several authors (18, 12) agree that interferon is not produced during the LCM virus infections of mice

The relative specificity of the inhibitory influence upon the stem cells was suggested by the almost unimpaired function of the immunocompetent cells The generation of very large numbers of plaque forming cells in the spleens of the acutely infected mice (Table 5) was not consistent with any comprehensive inhibition of cellular differentiation and proliferation

Interference with haemopoietic stem cell function has also been demonstrated in the course of acute infections with the Friend, Rauscher (13) and ectromelia (8) viruses These infections are, however, associated with stimulation of haempoiesis The Friend

and Rauscher viruses cause enhancement of the erythropoiesis, and the ectromelia virus produces increased levels of the colony-stimulating factor in the serum

As yet we have no data concerning the recovery of endogeneous stem cell function in mice acutely infected with LCM virus It is notable, however, that overt clinical signs of the heamopoietic disorder were observed only in mice exposed to X-irradiation, i e mice in urgent need of a haemopoietic stem cell response (3) It is probable that the complete reservoirs of more mature haemopoietic cells in the unirradiated mice rendered them protected until recovery of the stem cell function had occurred In this context it may be mentioned that adult mice exhibit limited demands for haemopoietic stem cells compared with the demands at the time of birth Considerable differences have been recorded in both the mitotic activity (2) and the migration (1) of stem cells Accordingly, more serious consequences may be expected to result from a viral interference with the stem cells during the neonatal period, that is in the case of the prospective virus carriers

As regards the adult virus carriers their numbers of colony forming stem cells were decreased by some 50 per cent, and this finding seemed to agree with their relative increase in sensitivity to X rays (3) The experiments did not, however, indicate any inhibitory influences on the function of haemopoietic stem cells in the adult virus carriers For the reasons mentioned above it seems likely that their haemopoietic defects are already induced during the neonatal period by a viral effect similar to that observed with the mature acutely infected mice The interference with haemopoiesis in early life might perhaps also be relevant to the obscure deaths of a considerable number of infected babies (6)

An intimate relationship may exist between the haemopoietic effect and the most characteristic feature of the LCM virus, namely its ability to induce immunological tolerance in newborn mice While available immunocompetent cells in adult mice may afford a prompt antiviral response, the immune apparatus of babies is very immature and dependent on a generation of antigen reactive cells from precursor cells Using chromosome marker techniques *Wu et al* (19) and *Trentin et al* (16) have convincingly demonstrated that these precursor cells are identical with, or at least very closely related to the haemopoietic colony-forming stem cells That is, the viral effect upon the colony forming cells of newborn mice may be expected to cause serious defects in the function of the immune system One could imagine a deficiency of the precursor cells, or a profound interference with their proliferation and differentiation in the thymus, analogous to the inhibition of haemopoietic colony formation in the spleen and bone marrow It therefore seems reasonable to suggest that the viral stem cell effect may be a very important factor in the induction of immunological tolerance by the neonatal inoculation of LCM virus

The same arguments can also explain the results obtained with ALS treated adult mice These mice are also readily rendered persistent, tolerant virus carriers by inoculations of LCM virus Even a short treatment with ALS for not more than six days has been shown to be sufficient (7) The ALS treatment deprives the animals of many of their mature immunocompetent cells and like the babies the development or recovery of their immunocompetence is very dependent on *de novo* generation of mature cells from the precursor cells i e the stem cells

It is tempting to speculate that the viral effect on the haemopoietic stem cells may endow the LCM virus with a selective advantage of survival in the host If this is so, then one could imagine that a similar influence might be exercised by other viruses capable of causing persistent tolerant infections

The authors are indepted to M[illegible] Radiofysisk Laboratorium F[illegible] for providing X irradiation facilit[illegible] ex

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A MICRO ASSAY FOR MOUSE AND HUMAN INTERFERON

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A simple micro assay for testing mouse and human interferon is described The L-F$_1$ line of mouse lung fibroblasts and the U-line of human amnion cells were seeded in microtrays, treated with two fold dilutions of 0 1 ml interferon and challenged with Vesicular Stomatitis virus Reading was done microscopically for presence of cytopathogenic effect and macroscopically for metabolic activity indicated by colour change Sensitivity of the test is comparable to the commonly employed plaque reduction assay and the macro infectivity inhibition test Reproducibility of the system is satisfactory The interferon titres are influenced by the age and density of cells length of interferon treatment and the dose of challenge virus Age, density and length of treatment are standardized for the test and by means of a standard slope it is possible to adjust interferon titres to a standard challenge dose The micro system is time saving, technically simple and allows testing of extremely small volumes of test material

Several different methods have been described for testing interferon (IF) (3) The most frequently employed methods are the inhibition of viral cytopathogenic effect (CPE) in tube cultures (7, 13), reduction of plaque counts on a monolayer of cells under an agar overlay (2, 17) and the yield reduction assay (5, 6) The dye uptake method, an indirect quantitation of CPE, designed by *Finter* (4) is also employed in several laboratories These methods are time consuming and expensive in terms of test material, Even more so are the methods based on assay of production of viral nucleic acid There is a need for simple methods requiring small amounts of test materials, especially for screening large numbers of samples

Since *Takatsy* (15) developed the calibrated spiral loops for preparation of serial dilutions in micro volumes, the micro technique has been applied to an ever increasing number of biological tests The method and the equipment have been further refined and today it is routinely used in most microbiological laboratories for different serological tests, including tests for biological activities in tissue cultures A micro method for testing human and chick IF, based on inhibition of CPE development, was introduced by *Tilles & Finland* (16) A micro modifi of the dye binding assay scribed for rabbit IF by micro methods were extremely econ

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In the present communication we describe a simple modified micro technique for testing mouse and human IF, and the most important factors influencing the reproducibility of the method

MATERIALS AND METHODS

Cells The L F_1 line of mouse fibroblasts was given to us by Dr *S Haahr* Aarhus University Denmark It was grown in Eagle s minimal essential medium (MEM) with Hanks salts (Grand Island Biological Co San Francisco Calif, GIBCO) supplemented with 10 per cent inactivated calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium with 2 per cent inactivated calf serum and 0 132 per cent $NaHCO_3$

The U line of human amnion cells was obtained from Dr *K Cantell* State Serum Institute Helsinki Finland The cells were grown and maintained in the same medium as the L F_1 cells.

The Vero line of African Green Monkey Kidney cells was obtained from Dr *J C Ulstrup* Ullevål Hospital Oslo It was grown in medium 199 with Hanks salts (GIBCO) supplemented with 5 per cent inactivated calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 2 per cent inactivated calf serum and 0 132 per cent $NaHCO_3$

Human Skin Fibroblasts (HSF) were received from Dr *H Bondevik* Rikshospitalet Oslo They were grown in medium 199 with Hanks salts supplemented with 20 per cent inactivated calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 10 or 5 per cent inactivated calf serum and 0 132 per cent $NaHCO_3$ For IF assay the same medium with 2 per cent inactivated calf serum was used All titrations included in this study were done with passage numbers 19 or 20

Virus Vesicular Stomatitis virus (VSV) Indiana strain was used as challenge virus It was passed two times in the allantoic cavity of embryonated hens eggs in our laboratory and then several times in L F_1 cells Monolayers of L F_1 cells were inoculated with VSV at a multiplicity which gave a pronounced CPE after 24 hours of incubation at 37° C Then the cultures were frozen and thawed three times and the supernatant cleared of cellular debris by centrifugation at 3000 rpm for 10 minutes The supernatant fluid was stored at −70° C Infectious titre was estimated by *the end point titration* method in tissue culture tubes and in microtrays The plaque titre was assayed in monolayers of L F cells and L-cells in 60 mm plastic petri dishes (Nunclone, Nunc Roskilde Denmark) The dishes were inoculated with 0 2 ml of serial dilutions of VSV and incubated at *37° C* for one hour Then the cells were overlayed with 10 ml of a 1 per cent *methocel (MC 4000* cp, *Fluka)* in medium 199 supplemented with 2 per cent inactivated calf serum and 0 132 per cent $NaHCO_3$ After 2 3 days of incubation at 37° C in 5 per cent CO_2 atmosphere clear plaques could be observed microscopically The overlay was then removed and the *cells fixed with a 4 per cent solution of* formaldehyd for 15 minutes After rinsing the monolayers with tap water they were stained with Giemsa

Herpes virus type 1 Tyler strain was received from Dr *P Leinikki* University of Helsinki Finland It was passed 6 times in Vero cells in our laboratory Monolayers of Vero cells were inoculated to give a strong CPE within three days at 37° C The cultures were then frozen at —20° C over night thawed and centrifuged for 10 minutes at *3000* rpm The clear supernatant after addition of 10 per cent rabbit serum was stored in 0 5 ml amounts at —70° C Infectious titre was estimated by the end point titration method in *microtrays using Vero or HSF cells 20 000 per cup*

Interferon (IF) Standard mouse IF was received from the National Institute of Health (NIH) Bethesda Md USA It was diluted to contain 600 NIH standard mouse IF units per ml Human IF, produced in leukocyte suspensions was kindly given to us by Dr *K Cantell* It contained 20 000 units per ml IF samples were stored at —20° C

Microequipment Calibrated microdiluters and pipette droppers (Cook Engineering Co Alexandria Va USA) delivering 0 05 or 0 025 ml per drop were used in the microtests They were rinsed in distilled water and boiled for 15 minutes before use Linbro IS FB 96 flatbottomed micro trays (Linbro Chemical Industries New Haven Conn USA) were sterilized by immersing in 96 per cent alcohol for one hour and allowed to dry under UV light over night

Assay for Antiviral Activity

Microassay for human and mouse interferon Each cup in the tray was seeded with 0 1 ml of a cell suspension containing 20 000 U cells or 40 000 L F_1 cells (if not otherwise indicated) Two-fold dilutions of IF were added 0 1 ml per cup 6–8 cups for each dilution The controls received 0 1 ml of medium instead of interferon The tray was then dried on the top surface with a steril cloth and sealed with cellotape by use of a tape dispenser *After incubation over night at* 37° C 10 tissue culture infecting doses ($TCID_{50}$) of VSV in 0 025 ml of medium were added to each cup (if not otherwise indicated) In some

cases the medium was changed prior to addition of virus as indicated in the results. Back titration of virus was included in each test. The tray was then dried and sealed again with cellotape and incubated further at 37° C. After two days of incubation the medium had turned yellow due to cellular metabolism. The tape was then removed and the tray covered with flame sterilized aluminium foil and placed in the 37° C incubator for 2–3 hours. When all cups had turned red following CO exchange with the open air, the tray was sealed again and incubated over night. The next day the test was read microscopically for presence of CPE and macroscopically. Cups containing well preserved monolayers controls and protected cells continue to metabolize, and consequently the pH of the medium decreases shown by yellow color of the indicator. In contrast the cups in which cells were destroyed by the virus attack remained alkaline and red (10). The titre was estimated as the dilution which inhibited destruction of the cells in 50 per cent of the cups calculated by the method of Reed and Muench.

Plaque reduction method. For assay of mouse IF 60 mm *plastic* petri dishes were seeded with 10^6 L-F_1 cells in 10 ml of medium. After two days in a CO_2 incubator when a monolayer had been formed, the medium was removed and 2 ml of *two-fold* dilutions of mouse IF added, two dishes per dilution. Virus and cell control dishes received 2 ml of medium. After over night incubation at 37° C in 5 per cent CO atmosphere the IF dilutions were removed by suction and 0.2 ml of VSV dilution containing approx. 100 plaque forming units was added. The dishes were incubated at 37° C *for* one hour and then overlayed with 10 ml of a one per cent methocel. After 3 days of incubation the cells were fixed and stained as described. The IF titre was calculated as the dilution which reduced the plaque count by 50 per cent compared to the virus controls.

For assaying human IF by the plaque reduction method 2 ml of medium *containing* 10^6 U cells were pipetted into each dish. Two-fold dilutions of human IF were added immediately after the cells two *dishes* per dilution. After over night incubation at 37° C in a CO_2 incubator the medium containing IF was removed by suction. The dishes were challenged with VSV and processed further as described for mouse IF.

Infectivity inhibition test for assaying mouse and human IF was performed in tissue culture tubes. These were seeded with 10^5 cells in 1 ml of medium. After 2 days the medium was decanted and 0.5 ml of two fold serial dilutions of IF was added to each tube, two tubes per dilution. The tubes were incubated in a roller drum at 37° C over night, then the IF was decanted and the monolayers challenged with 10 $TCID_{50}$ of VSV in 1 ml of medium per tube. The tubes were incubated in the roller drum for three more days before microscopical examination for presence of CPE. The IF titre was estimated as the dilution which inhibited the viral CPE in 50 per cent of the tubes, calculated by the method of Reed and Muench.

RESULTS

Influence of Cell Density and the Age of Cell Cultures on Sensitivity to IF

On three subsequent days microtrays were seeded with 1×10^4, 2×10^4, 3×10^4, 4×10^4 or 5×10^4 L-F_1 cells per cup in 0.2 ml of medium, one tray per cell concentration, and allowed to form monolayers. On the fourth day 5 trays were seeded again with the same number of cells in 0.1 ml of medium. The medium from the 1–3 days old cells was decanted and 0.1 ml of fresh medium was added to each cup. Two-fold dilutions of mouse IF, 1/30 to 1/960, were added to both the freshly seeded and the 1–3 days old trays 0.1 ml per cup and 8 cups per dilution. The control cups were given 0.1 ml of medium. The trays were sealed and incubated over night, then challenged with VSV as described. The results of this experiment are summarized in Table 1. Increasing IF titres were demonstrated with increasing cell density and possibly to some extent with increasing age of cultures. Two and three days old cells, with a cell density higher than 10^4 cells per cup degenerated before the completion of the test, and also the younger cultures seeded with 5×10^4 cells showed some degeneration at the time of final reading. In the light of these results 4×10^4 L-F_1 cells per cup were employed in the further experiments, and the cells were treated with IF in suspension immediately after seeding.

Preliminary studies indicated that with the present method U-cells did not survive the extended period of culturing required in the experiments employing cells seeded several days prior to IF treatment. Therefore, in the following experiments, cells were treated with IF immediately after seeding. The number of cells used per cup was based on the following

observations 1×10^4 U cells per cup did not consistently form monolayer when the test was examined, which made the reading more difficult 5×10^4 or more cells per cup did not survive the observation period Cups seeded with 2×10^4 and 4×10^4 cells gave consistently satisfactory end result In the following experiments therefore, cups were seeded with 2×10^4 cells as this in our hands resulted in somewhat higher titres compared to 4×10^4 cells per cup

TABLE 1 *Influence of the Age and Density of Cells on the Titre of Mouse Interferon Assayed in L F₁ Cells*

Age of cells after seeding	Number of cells per cup 1×10^4	2×10^4	3×10^4	4×10^4	5×10^4
0 (cells in suspension)	2 5*	2 7	2 7	2 9	3 1†
1 day	2 5	2 7	2 9	2 9	2 6†
2 days	2 6	§	§	§	§
3 days	2 8	§	§	§	§

* $\log_{10}$ interferon units per 0 1 ml
† Some degeneration before the final reading
§ Degeneration before completion

Influence of the Duration of Interferon Treatment of the Cells

Interferon titre depends to a large extent on the duration of the incubation of cells with IF In most studies 24 hours of incubation has been used but shorter time has also been reported to be preferable (8 17) To evaluate the optimal time of incubation for our test systems mouse IF was assayed in three parallel microtrays and after 5 24 or 48 hours of treatment the cells were challenged with 10 TCID of VSV Another tray was treated with IF for 24 hours then the medium was transferred cup by cup to a new tray seeded with 40 000 L F_1 cells per cup After 24 hours of incubation the tray with transferred medium was challenged with virus The results of two experiments are given in Table 2 Incubation for 24 hours resulted in higher IF titre than that after 5 hours of incubation This titre was not increased significantly by further incubation The IF containing medium removed after 24 hours contained only small amounts of viral inhibitor Comparable inhibition was also observed with medium removed from normal L F_1 cells after 24 hours of incubation

TABLE 2 *Mouse Interferon Assayed in L F₁ Cells with Different Duration of Interferon Treatment*

	Duration of interferon treatment 5h	24h	48h	24h†
Experiment 1	1 37*	1 90	1 55	1 20
Experiment 2		1 67	1 87	0 94

* $\log_{10}$ interferon units per 0 1 ml
† Interferon dilutions transferred after 24 h incubation with cells

TABLE 3 *Influence of the Continuous Presence of Interferon in the Medium after 24 Hours Treatment on the Titre of Mouse and Human Interferons*

		Titre of interferon: Interferon present after 24 hours	Interferon removed after 24 hours
Mouse Interferon	Experiment 1	2 22*	2 30
	Experiment 2	1 82	1 85
	Experiment 3	2 10	1 85
Human Interferon	Experiment 1	3 13	3 17
	Experiment 2	3 32	3 30

* $\log_{10}$ interferon units per 0 1 ml

As significant IF activity was no longer demonstrable in the medium after 24 hours of incubation we examined the necessity of removal of IF dilutions before addition of challenge virus Two fold dilutions of IF were added of two parallel trays as previously described After 24 hours of incubation the IF was decanted from one of the trays and replaced with 0 2 ml of fresh medium then both the trays were challenged with VSV and the development of viral infection registered In Table 3 the results of three repeated experiments are tabulated There are

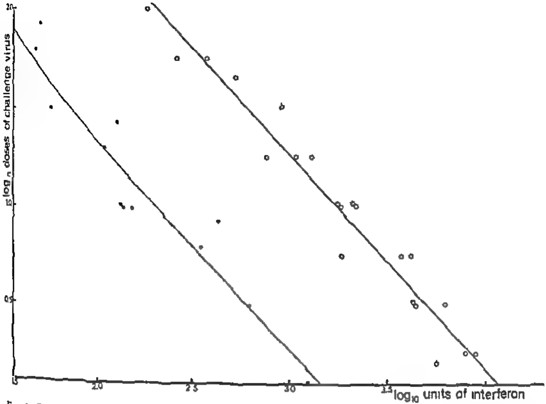

Fig 1 Dependence of the interferon titre on the dose of challenge virus used (Closed circles represent mouse interferon in L F_1 cells open circles represent human interferon in U cells)

no significant differences in titres between the tests with or without removal of IF after 24 hours treatment. Similar results were obtained in the experiments with human IF

Influence of the Dose of Challenge Virus on the IF Titre

Mouse and human IF were assayed by the micro method against various doses of challenge virus The results of several experiments are illustrated on Fig 1 The regression lines were computed as the least squares line A correlation coefficient of < -0.99 indicated a straight line relationship between $\log_{10}$ IF titre and $\log_{10}$ units of challenge virus employed, within the range of the applied doses The slope for the regression lines is practically identical for human IF (-1.094 ± 0.19) and for mouse IF (-1.099 ± 0.26) with the 95 per cent confidence limit computed under the assumption of a t distribution

TABLE 4 *Influence of Variation of the Challenge Virus Dose on Interferon Titre in Different Cell/Virus Combinations Comparison of the Slopes for Regression Lines*

Interferon	Cell/virus	Slope for regression line
Mouse	L F_1/VSV	-1.099 ± 0.26*
Human	U/VSV	-1.094 ± 0.19*
Human	HSF/Herpes	-0.66 ± 0.29†
Human	Vero/Herpes	-0.79 ± 0.28†

* Within 95 per cent confidence limit
† Within 95 per cent confidence limit

Assay of Human IF in Different Cell/Virus Systems

In order to compare the slope of the regression lines when the same IF is tested in a different cell/virus system human IF was assayed in Vero and HSF cells and challenged with Herpes virus type 1 Unfortunately

TABLE 5 *Comparison of the Titres of Human and Mouse Interferons Assayed by the Plaque Reduction, Micro and Macro Infectivity Inhibition Methods*

		Plaque reduction units/2 ml	Infectivity inhibition Macro units/0 5 ml	Infectivity inhibition Micro units/0 1 ml
Mouse interferon	Experiment 1	2 6* (1 3)	2 2 (1 5)	2 15
NIH titre	Experiment 2	2 5 (1 2)	2 15 (1 45)	2 2
1 8/0 1 ml	Experiment 3	–	[illegible]48 (1 78)	1 15
Human interferon	Experiment 1	3 2 (1 9)	3 8 (3 1)	3 35
Cantells titre	Experiment 2	3 5 (2 2)	4 1 (3 4)	3 27
3 3/0 1 ml	Experiment 3	–	3 7 (3 0)	3 35

* $\log_{10}$ units of interferon
Figures in brackets are IF titres calculated in $\log_{10}$ units per 0 1 ml

our Herpes 1 strain did not produce clear CPE in U cells even after several passages thus these cells could not be included in this comparison Vero or HSF cells were seeded in microtrays, 20 000 cells per cup, treated with serial dilutions of human IF, and after 24 hours of incubation challenged with increasing doses of Herpes virus The cultures were examined for CPE after 3 days and IF titres calculated as usual The resulting regression lines were computed as the least squares line In Table 4 the calculated slopes are compared with those found for human IF assayed in the U cell/VSV system and with mouse IF assayed in the L-F, cell/VSV system The results suggest that the slope of the regression line depends on the challenge virus, while the interferon and the cell system have only minor influence

Sensitivity of the Micro Assay Compared to the Plaque Reduction Method and the Macro Assay for Infectivity Inhibition

Samples of human and mouse IF were tested simultaneously with the different methods The results are tabulated in Table 5 Per identical volume units the micro assay for human IF seems to be 20 times more sensitive than the plaque reduction method and as sensitive as the infectivity inhibition macro method The titre in our system is the same as reported by *Cantell* For mouse IF the micro method seems to be almost 10 times more sensitive than the plaque reduction and 5 times more sensitive than the infectivity inhibition macro method when expressed in identical volume units One NIH unit is equivalent to 2 2 units assayed with the micro system

Microscopical examination of the monolayers and the reading for colour changes gave comparable results both with human and mouse IF (Table 6) Macroscopical evaluation of the borderline cups in a few cases presented some difficulties

TABLE 6 *Titres of Human and Mouse Interferons Assayed by the Micro Test Comparison of the Titres Obtained by Macroscopical and Microscopical Reading*

		Titre of interferon Macro (metabolism)	Titre of interferon Micro (CPE)
Human interferon	Experiment 1	3 20*	3 27
	Experiment 2	3 27	3 17
Mouse interferon	Experiment 1	2 15	2 15
	Experiment 2	1 85	1 85

* $\log_{10}$ units of interferon per 0 1 ml

DISCUSSION

The main advantage of the micro assay is that it works with extremely small volumes of test material. This makes possible testing of individual samples from small experimental animals and also small clinical samples from humans without undesirable dilution of the samples. It is also time saving compared with other test methods. For rapid screening the metabolic inhibition reading is extremely convenient, but even microscopical examination of the small cups is not very time consuming. We prefer microscopic reading, thereby avoiding occasional errors introduced by technical failures, for example leakage under or through the sealing tape. The present test system is further simplified compared with previously described micro assays (14, 16). The time saving method of introducing the virus in the presence of IF can only be used in systems, where absence of non interferon viral inhibitors is ensured. The sensitivity of the micro assay is fully comparable with that of the plaque reduction test and macro assay for infectivity inhibition.

In all IF assays a number of factors influencing the sensitivity of the test are variable, such as number of cells per test unit (3, 11), cell age (1, 3, 9, 12), the length of incubation with IF (3, 8, 17) and the number of infective virus particles in the challenge dose (3). Among these cell density, age of cell cultures and duration of IF treatment are always controlled and standardized for each test. Our findings on the effect of these variables on IF titre are generally in agreement with earlier observations. Small variations of the challenge virus titre cannot as easily be avoided. However, as all IF micro assays include back titration of the challenge virus is it possible to calculate the IF titres to a standard challenge dose by means of the slope of the regression line of the cell/virus system. The reproducibility of the micro assay is fully satisfactory taking advantage of this calculation. Inter dependence of the slope of the regression line on various factors involved in the test system is an interesting side product of this study. Further examination of the problem is in progress.

The micro assay has to date been tested for human and mouse IF, but we cannot see any reason why it should not be applicable for testing IF from other animal species too.

The excellent technical assistance of Mrs *S Beck* is grateful1y acknowledged.

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GRAFT-VERSUS-HOST ACTIVITY OF RAT LYMPHOCYTES AFTER SENSITIZATION *IN VITRO*

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In order to test the hypothesis that the small increase in graft versus host (GVH) activity after immunization *in vivo* against strong histocompatibility antigens may be due to an inhibitory effect of allo-antibody on the cellular immune response rat lymphocytes were sensitized *in vitro* with allogeneic lymphocytes Peripheral lymphocytes from inbred Fischer (F) strain rats were cultured *in vitro* either alone or together with (F×BN) F1 lymphocytes in conditions where humoral antibodies are not demonstrably formed After 4 and 6 days of culture the activity of the stimulated and non stimulated cells was compared in a GVH assay using (F×BN) F1 and (F×AS2) F1 hybrids as hosts The activity of the *in vitro* sensitized cells against (F×BN) F1 recipients after four and six days of culture was not significantly enhanced These findings support the alternative hypothesis that a very high proportion of cells are in fact sensitive to histocompatibility antigens, even in the non immune animal

As a result of experiments in which mice (12), chickens (15), and rats (6) were immunized with allo antigens, an inverse relationship between antigenic strength and the factor of immunization (F I) has been demonstrated as measured in a graft versus host (GVH) assay, i e the stronger the antigen the less the difference between a normal and an immune population of lymphoid cells

The original suggestion (12) was that the proportion of antigen sensitive cells recognizing strong allo antigens is very high, even in the non immune animal and therefore can not be substantially increased by immunization

An alternative possibility (11) is that the humoral antibody response to strong allo-antigens inhibits the development of the cellular immune response

In an attempt to distinguish between these two possibilities I have sensitized rat lymphocytes in mixed leucocyte cultures (MLC) where the formation of allo antibodies is unlikely Furthermore the possibility that sensitized cells or their progeny migrate to other organs prior to the GVH assay is eliminated in this system

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METHODS

Rats

Donors of the responding cells were of the inbred Fischer (F) strain

TABLE 1 *Graft versus Host Assay Blood Leucocytes Sensitized in Vitro against (F×BN)F1*

Exp No	Potency ratio (F×BN)F1 recipients	cpm in mixed cultures F+F×BN	Ratio cpm in mixed cult / cpm in unmixed cult
1	5 5	980	20
2	0 76	1416	3
3	0 88	970	14
4	1 1	813	16
5	1 5	1330	21
6	1 9	2146	29
Median	1 3	1155	18

The potency ratio is not significantly different from 1 0 (Wilcoxon's signed rank test)

Donors of the stimulating cells in MLC were (F×BN)F1 Recipients in the GVH assays were the F1 hybrids F×BN and F×AS2 the latter serving as specificity controls

F BN, and AS2 are all AgB different The F I after immunization *in vitro* in the combination F to (F×BN)F1 varies between 0 7 and 1 4 (6 and author s unpublished observations)

Leucocyte Cultures

Blood leucocytes were cultured as described by *Sorensen et al* (1971) with the modification that the culture vials were 100 ml infusion flasks and that the culture volumes were 10–15 ml Equal numbers of parental and F1 lymphocytes were mixed The culture period was 4 days

Thoracic duct lymphocytes (TDL s) were obtained by the method described by *Gowans* (1959) The collecting flasks were immersed in a cooling bath at 4° C The collections were made for up to 18 hours during the first 3 postoperative days The TDL s were washed three times in Eagle s MEM (Flow Laboratories Irvine Scotland) The concentration was adjusted to $3 0\times10^6$/ml and 20–25 per cent of blood leucocytes at the same concentration were admixed* Ten parts of parental cells and one part of F1 cells were mixed and cultured for 6 days The medium which was supplemented with streptomycin 100 μg/ml was changed twice

At the end of the culture period 500 μl aliquots were removed and incubated with 0 1 μCi ^{14}C thymidine 60 mCi/mMol (The Radiochemical Centre, Amersham England) for 4 hours The thymidine incorporation was determined according to *Sorensen et al* (1971) If the difference between the mean uptake in the mixed cultures and the highest of the unmixed cultures was below 200 cpm, the stimulation was considered insignificant and the experiment was discarded

After 4 or 6 days of culture viable cells were counted with the aid of trypan blue The unmixed cultures of F and (F×BN)F1 cells were now mixed for the GVH assay in the same ratio as the cells in the mixed cultures

GVH Assay

The lymph node weight assay described by *Ford et al* (1970) was used Two or three cell doses ranging from 3×10 to 6×10^6 viable nucleated cells were injected in a volume of 0 10 ml into the foot pads of (F×BN)F1 and (F×AS2)F1 rats 4–9 weeks old From two to six foot pads were injected with each dose Seven days later the draining popliteal lymph nodes were removed and weighed In the dose range employed the mean lymph node weight is linearly related to the dose of cells injected on a double log scale

The potency ratio which indicates how many more normal cells than immune cells are needed to give the same lymph node enlargement was calculated as described by *Simonsen* (1962 b) and is called the factor of immunization (F I)

Allo antibody Titration

Supernatants from mixed (F+F×BN) and unmixed (F) blood and TDL cultures were obtained after 4 and 6 days of culture

Haemagglutinating allo antibody was titrated using 3 fold dilutions in phosphate buffered saline To 2 drops of supernatant-dilution was added 1 drop of a 2 per cent erythrocyte suspension in 9 parts of 6 per cent Macrodex and 1 part of normal BN rat serum Macroscopic reading was performed after a 2 hour incubation at 37° C

* Since preliminary experiments had shown that addition of blood leucocytes would enhance the MLC responce of TDL's

TABLE 2 *Graft versus Host Assay Thoracic Duct Lymphocytes Sensiti ed in I itro against (F×BN)F1*

Exp No	Potency ratio (F×BN)F1 recipients	Potency ratio (F×AS2)F1 recipients	cpm in mixed cultures F+(F×BN)F1	Ratio cpm in MLC / cpm in unmixed cult
1	1 5		1621	10
2	1 4		427	5
3	2 7		938	3
4	0 78	0 87	891	2
5	2 0	1 3	893	4
6	1 1	1 0	723	2
7	0 96	0 78	859	3
8	1 2	0 89	792	4
9	0 71	0 60	840	2
Median	1 2*	0 89*	859	3

* Not s gn f cantly d fferent from 1 0 Neither is 1 2 different from 0 89 (W lcoxon s s gned rank test)

TABLE 3 *Concentration of V able Cells in Cultures Used for GVH Assays (× 10⁻⁶/ml)*

Cells cultured	Blood cells (n=5) Mean	Range	TDL s (n=9) Mean	Range
F unmixed	1 2	0 72 – 1 8	0 61	0 43 – 1 1
(F×BN)F1 unmixed	0 89	0 72 1 2	0 62	0 44 – 0 84
F+(F×BN)F1	1 2	0 84 2 1	0 63	0 45 0 84

The blood cells have been cultured for 4 days and the TDL s for 6 days
In both cases the in t al concentration was 3 0×10 lymphocytes per ml

RESULTS

Blood leucocyte cultures Leucocytes from F and F×BN were cultured for 4 days unmixed as well as mixed in equal proportions The results are shown in Table 1 There is a slightly enhanced GVH activity of the cells from the mixed cultures but the potency ratio is not s gnificantly different from 1

The ability of the cultured cells to produce lymph node enlargement was comparable to that of fresh blood leucocytes although the slope of the dose response curve was a little less

TDL cultures In order to minimize the effect of a possible cytotoxic killing of the F1 cells on the proportion of parental to F1 cells during the culture period a ratio of 10+1 of F to F×BN cells was used This still gave a significant transformation

The ability of the cultured cells to give lymph node enlargement was a little smaller than that of fresh TDL s but the slopes were identical

The results of the GVH assays are presented in Table 2 which shows that there is a very slight increase in the GVH activity of cells sensitized *in vitro* against the BN antigens but the potency ratio is not significantly different from 1 Also in the third party controls (F×AS2)F1 recipients the GVH activity of cells immunized *in v tro*

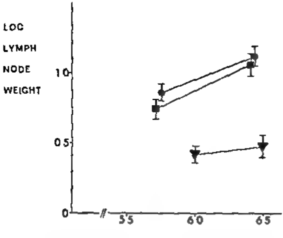

Fig 1 A typical GVH assay with cultured TDLs (Exp No 1 Table 2) F + (F×BN)F1 cells or (F×BN)F1 cells were injected into (F×BN)F1 rats
● Mean lymph node weight after injection of in vitro sensitized cells (F+F×BN)
■ Mean lymph node weight after injection of cultured non sensitized cells (F+F×BN)
▼ Mean lymph node weight after injection of cultured (F×BN)F1 cells
Bars indicate s e m

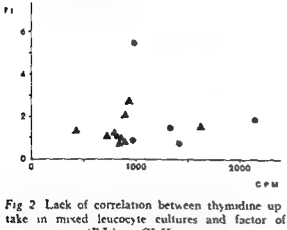

Fig 2 Lack of correlation between thymidine up take in mixed leucocyte cultures and factor of immunization (F I) in GVH assay
Kendall s coefficient of rank correlation + 0 20 not significantly different from 0
▲ TDL-cultures
● Blood leucocyte cultures

against the BN antigens was identical to the GVH activity of cultured, non sensitized cells (Table 2 column 3)

Table 3 shows the survival of cells in dif ferent types of cultures at the time of har vest The survival is higher in blood cultures harvested after 4 days than in TDL cultures harvested after 6 days, but there is no signif icant difference between the survival in mixed and in unmixed cultures

Figure 1 shows a typical GVH assay

Figure 2 shows that there is no correlation between the thymidine uptake in the mixed cultures and the potency ratio

Allo antibody titration Haemagglutinating antibodies were not demonstrable in either supernatants from mixed blood cell cultures or from TDL cultures after 4 or 6 days of culture

DISCUSSION

The possibility that the cellular immune re sponse to strong allo antigens is suppressed by humoral antibodies has previously been tested in this laboratory with two other techniques *Crone* (personal communication) compared with GVH assays the factor of immunization in normal and hormonally bursectomized chickens which are not able to mount an anti body response against the strong histocom patibility antigens of this species She found no difference in the F I *Ford* & *Simonsen* (1971) immunized rats incapable of an allo antibody response after 300 rads of X irradia tion with allogeneic cells They also found that the F I remained low after suppression of the allo antibody response

Elves (1969) has sensitized rat lympho cytes in MLC and measured their ability to mount a GVH reaction in a spleen test using AS as parental cells and (AS×AS2)F1 hybrids as stimulating cells in MLC, and in recipients in the GVH spleen test He found an increased GVH activity of the cells from the MLC However he did not control the specificity of the increased activity and the spleen test used by him does not estimate the relative potency of the stimulated cells to the unstimulated cells With these reservations it may be said that his data suggest a F I higher than that found in the present ex periments

Several authors have reported specific *in vitro* induction of cell mediated immunity in

mice against allo antigens (9, 10, 16) and in rats against hetero antigens (2) measured by target cell destruction Similar experiments have not been done with rat lymphocytes sensitized against allogeneic cells *in vitro*

Gordon et al (1967) cultured Lewis leucocytes with (BN×Lew)F1 leucocytes and injected the cells into Lewis rats bearing 2 day-old BN skin grafts They found accelerated rejection of the grafts in the rats receiving MLC cells They did not control the specificity of the reaction A similar experimental system was used by *Dyminski & Argyris* (1971) who found that C57Bl mouse lymphoid cells incubated with C3H macrophage monolayer would specifically accelerate the rejection of C3H skin grafts

The present experiments may be criticized because the culture conditions are suboptimal The thymidine incorporation in the mixed cultures in the 100 ml vials is only about 50 per cent of the uptake in the ordinary culture system using 2 ml vials Nevertheless, there is a large progeny of the cells activated in the MLC response Cell division starts after 48 hours but the daughter cells only seem to have little activity in the GVH reaction, since the potency ratio did not differ significantly from unity, and since there was no correlation between the thymidine uptake and the potency ratio

A comparison of the time course of the enlargement of lymph nodes draining immune and non immune lymphocyte populations has not been made in these experiments However, *Ford & Simonsen* (1971) using lymphoid cells immunized *in vivo* found that there was no difference in the tempo of the responses produced by the immune and non immune lymphocytes

It was not possible to demonstrate the production of antibodies to histocompatibility antigens in the mixed leucocyte cultures The sensitivity of the haemagglutination assay is not known exactly, but the serum titre 4 and 6 days after an *in vivo* primary immunization of F rats with (F×BN)F1 spleen cells was 1/3 to 1/9 (author s unpublished observations) *Alm* (1971), in experiments with chicken spleen cells, also found that no antibodies against histocompatibility antigens were produced in MLC

The conclusions of the present experiments are that the same small factor of immunization is found after sensitization *in vitro* against strong histocompatibility antigens as after immunization *in vivo*, and that an inhibition of the cellular immune response against allo-antigens by humoral antibodies therefore seems unlikely Furthermore, the majority of the cells activated in the MLC reaction seem to be inactive in the GVH reaction

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VARIANT OF THE B CHARACTER IN GROUP A_1B WITH ANTI-BH IN SERUM

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Blood grouping of a 65 year-old woman showed a normal A_1 antigen and in addition a weak B character, producing mixed field agglutination in anti B sera In her serum B and A substances were demonstrated and an anti BH antibody was found In the small family investigated, proposita was the only one who carried the abnormal blood group It is discussed whether the blood group variant is due to inheritance of a defective B gene or to the effect of a recessive suppressor gene

A weak B character on the red cells of a person of group A_1B with B substance in the serum together with an antibody, anti-BH, has been found Examination of the family revealed no such abnormality

MATERIAL AND METHODS

Case History

A 65 year-old woman (HJ) was admitted to Rigshospitalet, Copenhagen, from the County Hospital, Slagelse, because of attempted suicide She was unconscious and had anuria The day before the admission to Rigshospitalet she had received one unit of A_1B blood, regrouped at the Blood Grouping Department, Rigshospitalet In the blood grouping laboratory of the County Hospital Slagelse, she had been grouped twice as AB with a mixed field agglutination in the anti B testserum before blood transfusion and the erythrocytes of the donor blood were found compatible with her serum At Rigshospitalet blood samples were taken from her for blood grouping and crossmatching Although she was dialysed she died with anuria one week later, so it was only possible to get limited amount of blood samples for serological investigations

Serological Investigations

Methods 1 Routine investigation of the red cells Inculation of 2 drops of a 5 per cent red cell suspension with one drop of anti A and anti B test serum (DADE) for 45 minutes at room temperature

2 Incubation of the red cells with selected strong anti B test sera, some containing tartrazine dye some without dye, and with a number of other group A sera

3 Incubation of HJ red cells with serial dilutions of anti A and anti B test serum A_1B and A B cells as controls

4 Elution experiments a m *Landsteiner and Miller* (2) after incubation of HJ red cells with anti B resp anti A test serum, using A_1B and O cells as controls

5 Incubation of HJ cells with 2 different Coombs sera [illegible]

6 [illegible]

[illegible] from [illegible] immunized with *Shigella Shigae* vaccine (Statens Seruminstitut) and serum from a person of group O_h

7 Incubation of HJ serum with 12 group O, 5 group A_1 2 group A 6 group B, 2 group [illegible]

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TABLE 1 *Titres of Anti A (ORTH*

Cells	1	2	4	8	16
HJ	+++(+)	+++(+)	+++(+)	+++(+)	+++(+)
A_1B	+++(+)	+++(+)	+++(+)	+++(+)	+++(+)
A_2B	+++(+)	+++(+)	+++(+)	+++	++(+)

Titres of Anti-B (DAD

Cells	1	2	4	8	16
HJ	++	+(+)	+(+)	+	+
A_1B	++++	++++	++++	+++(+)	+++
A_2B	++++	++++	++++	+++(+)	+++

TABLE 2 *Elution of Anti B from HJ and Cont*

Eluate from	1	2	4	8	16	32
HJ cells	+++	+++	+++	+++	+++	++(+)
A_1B cells	+++	+++	+++	++(+)	++	+(+)
O cells	—	—	—	—	—	—

Tested with B cells

Elution of Anti-A from HJ and Control Ce

Eluate from	1	2	4	8	16	32
HJ cells	+++	+++	+++	++(+)	++	+
A_1B cells	+++	+++	+++	++(+)	++	+(+)
O cells	—	—	—	—	—	—

Tested with A_1 cells

and 2 group B cells from newborn babies, 10 group A_2B B group A B and HJ red cells for 10 minutes at 20° C in tubes followed by centrifugation for 1 minute at 500 g

8 Neutralization of anti BH from HJ with saliva from secretors One part of HJ serum and one part of saliva from an O secretor, a B secretor and an O non-secretor, respectively, were incubated for 30 minutes at 20° C After the neutralization the mixtures were tested against A_2B cells for 10 minutes at 20° C and centrifuged O and B cells served as controls

9 Establishment of presence of B substance in HJ serum· To serial dilutions of HJ serum serum from a B secretor and a B non-secretor respectively were added equal volumes of anti-B test serum (DADE) diluted 1 256 After incubation for 30 minutes at 20° C, the serum dilutions of HJ serum were tested against A_2B cells to avoid agglutination caused by the antibody in HJ serum The serum dilutions of serum from the B secretor and the B non secretor were tested against B cells The serum dilutions and the test cells were incubated for 10 minutes at 20° C and centrifuged for 1 minute at 500 g and then read As an extra control, undiluted HJ serum tested against A_1B red cells was included The presence of A substance in HJ serum was established likewise Anti B test serum was substituted with anti-A test serum (DADE) diluted 1 512, and after incubation the serum dilutions of HJ serum were tested against A_2B red cells, while the serum dilutions from an A secretor and an A non-secretor were tested against A_1 red cells

...against HJ A_1B and A_2B Red Cells

32	64	128	256	512	1024	2048
++(+)	++	+(+)	+	(+)	—	—
++(+)	++(+)	++	+(+)	+	(+)	—
++	+(+)	+	(+)	—	—	—

...against HJ, A_1B and A_2B Red Cells

32	64	128	256	512	1024	2048
(+)	—	—	—	—	—	—
++(+)	+(+)	+(+)	+	(+)	—	—
++(+)	+(+)	+(+)	+	(+)	—	—

...ells after Incubation with Anti B Test Serum (DADE)

64	128	256	512	1024	2048	4096	8192
++	+(+)	+(+)	+	+	(+)	(+)	—
+(+)	+	+	(+)	—	—	—	—
—	—	—	—	—	—	—	—

...after Incubation with Anti A Test Serum (DADE)

64	128	256	512
(+)	—	—	—
+	+	(+)	—
—	—	—	—

RESULTS

The reaction of HJ red cells in anti A test serum was like that of normal A_1B red cells The reaction of HJ red cells in all the anti-B sera tested was weak showing always the same picture of mixed field agglutination i e small agglutinates in the presence of large numbers of unagglutinated cells This also applied to sera without dye Before the blood transfusion the same reaction had also repeatedly been found at the blood grouping laboratory at the other hospital with another anti B test serum than DADE anti B together with a negative reaction of the serum with A and B test cells The result of the cross-matching had shown that the erythrocytes of the donor blood were compatible with HJ serum

The results are given in Tables 1–6

Table 1 shows the titre of anti-A and anti-B test serum respectively against HJ red cells and control cells It can be seen from the table that the strength of the A antigen of HJ red cells equals that of normal A_1B cells The strength of the B antigen on HJ red cells is considerably less than that of normal A_1B and A_2B cells

The titre of anti B in serum from A_1 per-

TABLE 3 *Investigation of the H Character of HJ Red Cells*

Ulex Europeus + cells from	1	2	4	8	16
HJ	—	—	—	—	—
A_1B	—	—	—	—	—
A_2B	+ + (+)	+ (+)	(+)	—	—
A_1	(+)	—	—	—	—
B	+ + (+)	+ (+)	(+)	—	—
O	+ + +	+ +	+	(+)	—
O_h	—	—	—	—	—
Chicken serum + cells from					
HJ	(+)	—	—	—	—
A_1B	+	—	—	—	—
A_2B	+ +	+	(+)	—	—
A_1	+	—	—	—	—
B	+ +	+	(+)	—	—
O	+ + +	+ +	+	(+)	—
O_h	—	—	—	—	—
Laburnum + cells from					
HJ	—	—	—	—	—
A_1B	—	—	—	—	—
A_2B	+ +	+	(+)	—	—
A_1	(+)	—	—	—	—
B	+ +	+	(+)	—	—
O	+ + +	+ +	+	(+)	—
O_h	—	—	—	—	—

sons did not differ from the titre of anti B in serum from A persons if tested against HJ cells

It appears from Table 2 that an eluate from HJ cells contained anti B after incubation of HJ cells with anti B test serum and that the eluate contained more anti B than an eluate from normal A_1B cells Eluate from HJ cells contained almost the same amount of anti A as an eluate from normal A_1B cells after incubation of the cells with anti A test serum

HJ cells were negative by the direct Coombs test and they were not agglutinated by anti-T

It appears from Table 3 that HJ cells did not have more H upon their surface than normal A_1B cells

The other blood groups of HJ red cells were as follows *CDe/c e MSs* P_1, k/k Kp(a+), Fy(a–b+), Jk(a+b–) Le(a–b–), Lu(a–), I, Wr(a), Ve(a+) The reactions in the different test sera, except anti B showed no picture of mixed field agglutination

Investigations of HJ serum showed that it agglutinated only cells of group B, including group B cells from newborn babies (8 B cells tested), and of group A_2B (8 A_2B cells tested), and did not agglutinate cells of other ABO groups (12 O cells, 10 A_1B cells, 5 A_1 cells, 2 A_2 cells and HJ cells tested) HJ serum also reacted with group B and A B cells at 4°C but did not react at 37°C including indirect Coombs' technique, or with papainized and trypsinized cells followed by indirect Coombs' technique

Table 4 shows the result of titration of the antibody in HJ serum The titre of the antibody against A B and B red cells is the same (1 4)

TABLE 4 *Titration of the Antibody in HJ Serum against A_1B A_2B and O Cells at 20° C*

Ce ls	1	2	4	8	16
A B	—	—	—	—	—
A B	+(+)	+	(+)	—	—
B	+(+)	+	(+)	—	—
O	—	—	—	—	—

TABLE 5 *HJ Serum Neutrali ed with Saliva from an O Secretor and a B Secretor*

Serum from HJ + sal a from	Tested with cells of group		
	A_2B	O	B
O secretor	—	—	—
B secretor	—	—	—
O non secretor	+(+)	—	+(+)

Table 5 shows that it was possible to neutralize the antibody in HJ serum with sal va from an O secretor and a B secretor but not from an O non secretor

As it was not possible to obtain any saliva from HJ (her blood pressure was so low that her saliva glands did not function), HJ serum was investigated as to the presence of B and A substance

Table 6 shows that HJ serum contains B substance as well as A substance In contrast to HJ serum the serum of the donor, from whom she had received one unit of blood did not contain any detectable A or B sub stance

Investigation of HJ s Family

Fig 1 shows the results of the investigation of HJ s family HJ was divorced from her husband and it was not possible to obtain contact with him HJ is the only member of the family who has an abnormal serological pattern concerning the ABO system Anti H was not found in the sera of any of the members of HJ s small family investigated and anti B in the sera was quite normal ag glutinating red cells of group B A_1B as well as A B

DISCUSSION

It appears from the above data that HJ carries a weak B character in addition to a normal A_1 antigen on her red cells, but only little or no H In her serum an antibody is found which is characterized by agglutinat ing all B and A B red cells tested but not A_1B cells or her own cells Thus the antibody

TABLE 6 *Tests for the Presence of B and A Substance in HJ Serum*

B (DADE) diluted 1 256 + serum dilutions

	1	2	4	8	16	32	64	128	256	512	Tested with cells
ecretor	—	—	—	—	(+)	+	+(+)	+(+)	+(+)	+(+)	A B
non-secretor	—	—	—	(+)	+	+(+)	+(+)	+(+)	+(+)	+(+)	B
	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	B

A (DADE) d luted 1 512 + serum dilutions

	1	2	4	8	16	32	64	128	256	512	Tested with cells
ecretor	—	—	—	—	—	—	—	+(+	+	+(+)	A_2B
non-secretor	—	—		—	—	(+)	+	+(+	+(+)	+(+	A_1
	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+)	+(+	+(+)	+(+	A_1

serum undiluted + A_1B cells —

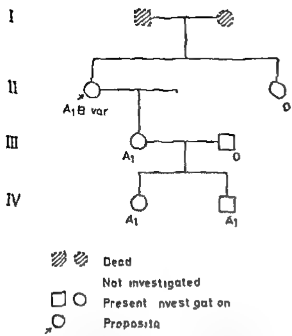

Fig 1 The HJ family I The parents of proposita II Proposita and her brother and sister III Daughter of proposita and her husband IV Their children The figures below the symbols The blood groups investigated

in HJ serum is directed against an antigen which has B characteristics as well as H characteristics and as the antibody can be neutralized by saliva from O and B secretors it must be anti BH It is a cold type anti body

HJ s sister is group O and her daughter and daughter s children are group A_1 HJ is the only person in the family investigated who carries a B character and this differs from normal in that her red cells give a weak agglutination presenting the picture of mixed field agglutination in all of the anti B test sera used Some red cells of group A B originating from the transfusion given prior to admission to Rigshospitalet must have been circulating in HJ s blood However the presence of these donor red cells has not been interfering with the investigations of HJ s blood as the agglutination picture of the red cells in HJ before and after the transfusion showed the same picture of mixed field agglutination if grouped with anti B testserum

Mollison (4) states that red cells with weak B antigens are characteristic in that they give more powerful eluates than normal B antigens after incubation of the red cells with anti B The eluate from HJ cells after incubation with anti B testserum contained more anti B than an eluate from normal A_1B cells thus indicating that the B antigen of HJ red cells is a weak B

It is not likely that she is a blood group chimera since no picture of mixed field agglutination is found by tests at her red cells against other anti sera with a view to determining her genotype Furthermore she is not a twin

Among others *Andersen* (1) has described a so called aquired B like antigen in addition to the A antigen on the red cells of some individuals harbouring anti B in their serum He described the phenotype as A^b The blood group of HJ is not A^b however as it differed from this blood group in that 1) the serum of HJ contains B substance while the serum of A^b persons does not 2) the serum of HJ contains anti BH agglutinins the serum of A^b persons contains anti B agglutinins

Sayfried et al (5) have described a family in which 3 members had weak B antigen in addition to the A_2 antigen, the serum contained anti B HJ does not belong to that group either Her red cells carry the A_1 antigen not the A antigen and her serum contains anti BH not anti B

For the same reason HJ does not belong to the blood group A_2B_x with anti B in serum described by *Madsen & Heisto* (3)

A possible explanation of the weak B character of HJ red cells is that HJ carries a defective B gen Unfortunately it has not been possible to group her parents because they are dead

It may be discussed whether the B variant of HJ is due to the effect of suppressor genes like yy which *Weiner et al* (6) have observed in A_m individuals However the A antigen of A_m red cells is not detectable with anti A test sera Furthermore A_m individuals

lack anti-A in their serum while HJ has anti-*BH in the serum*

Even though the family investigation gives no conclusive evidence, the possibility exists that a suppressor gene, recessive in character if present in double dose, may suppress the B character on HJ red cells Being A_1B^w and therefore capable of producing antibodies with anti H character as well as anti-B character, she has been able to produce the antibody anti-BH

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FINE STRUCTURE OF MELANIN-PRODUCING AEROMONADS

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The fine structure of *Aeromonas liquefaciens* and *Aeromonas salmonicida* grown in enriched and minimal media was examined by electron microscopy When the organisms were grown aerobically a larger amount of particles of high electron density was found than compared with cultivation under anaerobic condition The e particles were assumed to consist of melanin type of pigments The cell wall which was shown to consist of a distinct triple layered membrane is separated from a more indistinct triple layered cytoplasmic membrane by a relatively electron transparent periplasmic space of variable thickness Channels may occur through the cell envelope and the possible role of these channels in secretory functions of the cell is discussed Some large inclusions of low electron density which appeared in the cytoplasma are associated with storage products of the cell

Systems which form melanins occur widely throughout the phylogenic scale Most interest has been given to melanogenesis and the biochemical properties of the melanin producing cells of animals while less attention has been directed towards the melanin producing micro organisms However the pigments of *Aeromonas salmonicida* and *Aeromonas liquefaciens* have been shown to be of the melanin type as reported by *Griffin et al* (1953) *Ross* (1962) *and Aurstad* & *Dahle* (1972) The same type of pigments are also produced by some *Streptomyces* species (*Mencher* & *Heim* 1962 *Nitsch* & *Kutzner* 1968) and by a mutant of *Nadsoniella nigra* (*Ruband et al* 1969)

A close enzymoserological relationship between *Aeromonas liquefaciens* and *Aeromonas salmonicida* was demonstrated by *Dahle* (1969) and *Aurstad* & *Dahle* (1972) have shown that the pigment of *Aeromonas liquefaciens* has many chemical properties in common with the melanin produced by *Aeromonas salmonicida*

Melanins are still identified by ambigous tests which are based on phenoloxidases These enzymes are however not specific (*Yasunobu* 1959) and the structures of the pigments are quite uncertain

The electron microscope has proved to be a valuable tool for the investigation of melanin pigments of animal origin (*Gjesdal* 1959 *Breathnach et al* 1966) The aim of the present work was to use the electron microscope for the examination of some ultrastructures of the two melanin producing aeromonads *Aeromonas liquefaciens* and *Aeromonas salmonicida*

MATERIAL AND METHODS

Strains The strains used were *Aeromonas liquefaciens* ATCC 14715 and *Aeromonas salmonicida* ATCC 14174 obtained from the American Type Culture Collection Rockville (ATCC), Maryland U S A

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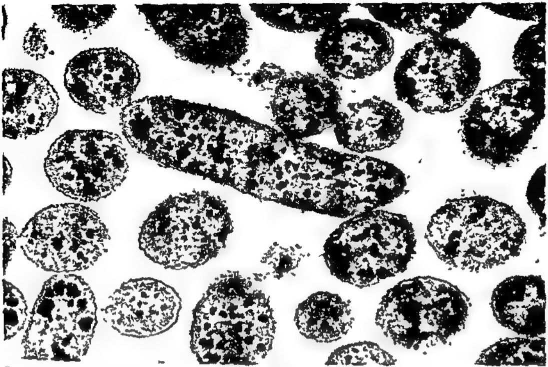

Fig 1 Electron micrograph of *Aeromonas liquefaciens* showing numerous particles of high electron density in the cells ($\times$ 30 000)

Cultural conditions Solid agar media (1 5 per cent agar) were used for the cultivation of the organisms Blood agar was prepared by including 5-7 per cent defibrinated bovine blood in nutrient agar (Difco*) Basal medium was prepared by mixing component solution with the agar The components in the solution were NH_4Cl, 5 g, NH_4NO_3 1 g, Na_2SO_4 2 g K_2HPO_4, 3 g, KH_2PO_4 1 g $MgSO_4$ H_2O 0 2 g, H_3BO_3 0 6 10^{-4} g $MnSO_4$ $4H_2O$, 0 8 10^{-4} g $CuSO_4$ 0 6 10^{-3} g $MNSO_4$ $4H_2O$ 0 8 10^{-3} g, $CuSO_4$ $5H_2O$ 4 10^{-4} g $ZnSO_4$ $7H_2O$, 88 10^{-3} g, glucose 10 g and DL glutamine 10 g The volume was adjusted to 1000 ml The media were sterilized at 121° C for 15 minutes and poured into sterile petri dishes The organisms were grown at 30° C for 42 hours in the laboratory atmosphere and also under anaerobic conditions

Preparation for electron microscopy Single colonies were harvested from the solid media by cutting out portions from the agar The colonies were fixed immediately by exposing them to cold 2 per cent glutaraldehyde in *Millonig's* buffer (1961) at pH 7 2 for 2 hour The material was then postfixed in 1 per cent osmium tetraoxide in *Millonig's* buffer (1961) at 4° C for 1 hour, and then dehydrated in increasing concentrations of alcohol, and embedded in Araldite according to the method of *Luft* (1961) Sections were cut with glass knifes using an LKB§ ultramicrotome collected on copper grids and stained with 5 per cent uranyl acetate (*Pease* 1964), and lead citrate (*Reynolds* 1963) Some sections were also studied without uranyl lead staining The sections were observed with Siemens‡ IA electron microscope operated at 80 kV using an instrument magnification of 15000 to 60000

* Difco laboratories Inc Michigan USA

§ LKB-Instruments Stockholm Sweden

‡ Siemens IA Germany

RESULTS

The most characteristic feature of the electron micrographs of *Aeromonas lique iens* and *Aeromonas salmonicida* grown on blood agar was the numerous particles of very high electron density, shown in Fig 1 and 2 The particles were especially numerous in the micrographs of *Aeromonas liquef... ens* also occurred in those of *Aeromonas ...icida* The shape and size of these

Fig 2 Electron micrograph of *Aeromonas sal monicida* An inclusion of low electron density with an indistinct membrane is located under the equator Some particles of the same electron den sity as shown in Fig 1 are also present (× 90000)

seemed to be similar in the micrographs of both the bacteria examined

In Fig 1 the diameters of the particles vary from ca 600 Å to ca 1500 Å The particles do not show any substructure and are not surrounded by a membrane The electron density within the separate particles seems to vary little even at high magnification (Fig 2) The sections made without uranyl-lead staining showed the same distinct particles of the same size, but with a slightly reduced electron density compared with the uranyl-lead stained sections The particles seem to be distributed throughout the cytoplasm Electron micrographs of *Aeromonas liquefaciens* grown anaerobically on the solid basal medium (Fig 3) show less electron dense particles as compared with Fig 1

In addition to the large particles of high electron density which were easily perceptible, small granules in the range 100 to 200 Å can be seen evenly distributed throughout the cytoplasm (Fig 2 and 5) Some of these particles seem to be spherical, while the majority are short rods

In the micrographs of *Aeromonas salmon icida* and *Aeromonas liquefac ens* shown in Fig 2 and 3 respectively some large in clusions with an indistinct membrane of 50–100 Å can be recognized The electron den sity of these inclusions are lower than for the surrounding cytoplasm The inclusions are nearly spherical with a diameter of 0 2–0 4 μ

The nuclear apparatus of the bacteria examined seems to be a dispersed network of fibrils of varying diameters enclosed within the area of the lowest electron density (Fig 1 and 2) The diameters seem to vary from 20 to 100 Å in diameter Direct contact be tween the nuclear material and the granular cytoplasm appears extensive at the interface between the two regions

The cell envelope of the *Aeromonas sal monicida* (Fig 4, 5 and 6) was seen to consist of three layers designated with the let

Fig 3 Electron micrograph of *Aeromonas lique faciens* grown anaerobically on solid basalmedium (× 30000)

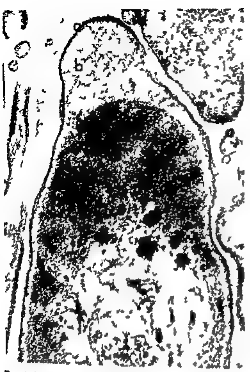

Fig 4 Electron micrograph of *Aeromonas salmonicida* showing the triple layer of the cell envelope the cell wall (a) the periplasmic space (b) and the cytoplasmic membrane (c) The periplasmic space varies greatly in thickness (× 90 000)

ters a b and ■ The layer ■ which is assumed to constitute the cell wall is a triple layered membrane consisting of two layers of high electron density separated by an electron transparent layer The total thickness of this triple layered membrane varies from 70 to 80 Å In some regions this triple layered membrane appears symmetrical (20 25 – 20 Å) but more often the outermost layer is somewhat denser and thicker (30 – 35 Å) than the innermost one (15 20 Å) (Fig 2 4 and 5) The layer b is of moderate electron density varying greatly in thickness (Fig 4 and 5) In Fig 6 this layer shows structural inclusions of high electron density of the same shape and size as the cytoplasmic particles described above (Fig 1 and 2) However layer b is usually without any structural inclusions as can be seen in Fig 4 and 5 Layer c which seems to constitute the cytoplasmic membrane possesses an indistinct triple layered structural organisation (Fig 5)

In Fig 5 channels may be seen originating in the cytoplasm and penetrating the bacterial envelope The channels appear in the micrographs of *Aeromonas salmonicida* as indistinct linear structures of low electron density with a diameter of 15–20 Å

DISCUSSION

Electron micrographs of melanin producing cells originating from vertebrates and micro organisms reveal characteristic structures of high electron density as a constant feature (*Taylor* & *Bagnara* 1969, *Jandi* 1966 *Bagnara et al* 1968 *Ruban et al* 1969) Although slight variations in the ultrastructure occur among the melanin producing cells of

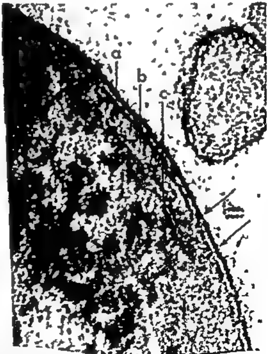

Fig 5 Details of the cell envelope of *Aeromonas salmonicida* The cell wall (a) consists of three layers as does the more indistinct cytoplasmic membrane (c) The cell wall and the cytoplasmic membrane are separated by the periplasmic (b) Channels penetrating the cell marked with arrows (× 240 000)

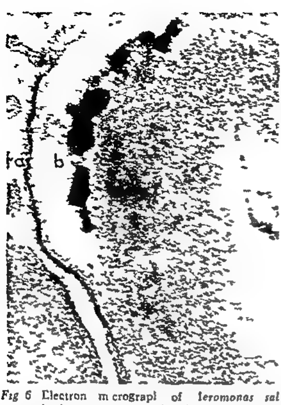

Fig 6 Electron micrograph of *Aeromonas salmonicida* showing granules of high electron density in the periplasmic space (b) which separates the cell wall (a) and the cytoplasmic membrane (c) (× 152 000)

vertebrates a constant finding is the presence of a melanin containing organelle the melanosome

The melanosome is an electron opaque spherical inclusion approximately 0.5 μ in diameter enclosed by a limiting membrane As described by *Ruban et al* (1969) the melanin pigment produced by a *Nadsoniella nigra* mutant also occurs as granules of small size with high electron density However these granules had no limiting membrane The pigments of *Aeromonas salmonicida* and *Aeromonos liquefaciens* have many properties in common with other pigments of melanin type (*Griffin et al* 1953 *Ross* 1962 and *Aurstad & Dahle* 1971) and the granules of high electron density shown in the micrographs of the aeromonads examined may be related to the production of the pigments of melanin type by these bacteria Furthermore the reduction of the electron dense particles in *Aeromonas liquefaciens* when grown anaerobically on a minimal medium is in accordance with the observation of *Aurstad & Dahle* (1972) that no visible pigment was synthesized by this organism under such conditions

The varying form and size of the granules within the sectioned bacteria may be explained in many ways One explanation is based on the fact that some of the granules are sectioned tangentially and therefore the shape size and electron density of the individual particles will vary within the sections Another explanation of the heterogeneity of the particles may be that they may represent different development stages However it has not yet been determined whether the degree of melanization is the only factor which determines the electron density of melanin containing organelles In the present micrographs no organized systems of internal membranes or fibers can be seen in the particles of high electron density as described previously in the formative stages of melanosomes of vertebrate cells (*Taylor et al* 1969)

The present observations demonstrate the existence of an asymmetric multilayered structure in the cell wall (designated with a in Fig 4 and 5) rather similar in dimensions and appearance to the more indistinct underlying cytoplasmic membrane (designated with c) This is in accordance with the electron microscopic observations of thin sections of other Gram negative bacteria (*De Petris* 1965 *Bladen & Waters* 1963) The two triple layered structures are separated by a fairly transparent periplasmic space of variable thickness (designated with b) Early authors considered this middle layer to be a technical artifact while later workers consider it as a real structural component of the bacterial cell wall (*Claus and Roth* 1964 and *De Petris et al* 1964)

The cavities observed in the periplasmic space which is formed by the cell wall and the cytoplasmic membrane (Fig 4) may be related to the formation and secretion of bacterial enzymes and toxins (*Smirnova et*

al 1971) The products to be secreted will be accumulated and enclosed within this periplasmic space and so separated from the cytoplasm and the external media After destruction of the cell wall the release of the metabolic products may occur without autolysis of the bacterial cell (*Smirnova et al* 1971)

Channels in the cell envelope of bacteria and fungi are described for a variety of organisms (*Manocha and Colvin* 1967, *Nermut* 1967, *Trevethick & Metzenberg* 1968), but the real significance of these structures is not known It can be assumed that they provide some means of communication between the cytoplasm and the external surface of the organisms, and *Smirnova* (1971) considers that enzymes produced within the cells pass through such channels to the exterior Whether the same mechanism also concerns the release of the electron dense particles is uncertain as no particles were observed in the channels but this possibility should be considered

The nearly spherical large inclusions observed in the micrographs of *Aeromonas salmonicida* and *Aeromonas liquefaciens* (Fig 2 and 3) may be explained as consisting of a substance of low electron scattering power Glycogen particles in bacteria are known to occur as small round "vacuoles" of rather diffuse outline and without a limiting membrane (*Cedergren and Holme* 1959) They seem to be empty but this appearance reflects the rather lowelectron scattering power of all polysaccharides of which glycogen is a representative Hydroxybutyric acid and neutral lipids are also known to occur as empty "vacuoles" in electron micrographs of bacteria (*Rose* 1968) These "vacuoles" are described as rather large with well defined limits, but with no real membrane separating them from the rest of the cytoplasm

All the small granules of the range 100 to 200 Å seen evenly distributed throughout the cytoplasm of the aeromonads seem to be identical to bacterial ribosomes or polysomes as described by *Kellenberger & Ryter* (1964), and the dispersed network of the nuclear apparatus in the bacteria examined corresponds to findings in other bacteria (*Conti & Gettner* 1961, *Caro* 1961)

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CHARACTERISTICS OF THE INHIBITION BY RABBIT IMMUNE SERUM OF THE BACTERICIDAL EFFECT OF CATTLE NORMAL SERUM ON *SALMONELLA TYPHIMURIUM* 395 MRO

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The bactericidal effect of cattle normal serum on *Salmonella typhimurium* 395 MRO is inhibited by highly avid rabbit anti-LPS (lipopolysaccharide) IgG Since the inhibitory effect was destroyed by pepsin, blocking of antigenic receptors on the bacterial surface by IgG is not the sole mechanism of inhibition Furthermore, bactericidal activity was interrupted by high concentrations of anti RO serum as late as after 20 min, and the inhibition was independent of the bacterial concentration, at least over a range of 2×10^{3} – 2×10^{9} bact/ml, which leads to the following hypothesis Anti LPS IgG bound to the bacterial surface activates the complement system without causing cell death but consumption of complement factors required for the slower bactericidal activity of normal serum

Normal serum from several mammalian animal species is bactericidal to many gram-negative bacteria, particularly the rough mutants of the family *Enterobacteriaceae* (4) This bactericidal effect, which depends on the complement system, is, for rough mutants of *Salmonella typhimurium* 395 MS, inhibited in the presence of rabbit hyperimmune serum (7) The extinction of the bactericidal activity is generally specific for the chemotype (6), which indicates that antibodies against the cell wall lipopolysaccharide (LPS) are involved After fractionation of rabbit immune serum on columns of DEAE cellulose and Sephadex G 200, only the IgG fraction showed clear-cut extinctive activity (25)

This extinctive activity resembles the Neisser Wechsberg phenomenon (23), a prozone phenomenon, where the bactericidal effect of an antibacterial serum is reduced at higher concentrations *Neisser and Wechsberg* (23) suggested that excess antibodies, uncombined with bacteria, deviated the complement activity, whereas *Coombs* (5) proposed that complement was consumed by soluble antigen-antibody complexes The mechanism of the extinction by IgG is not known Two main hypotheses are suggested (1) Specific blocking by the IgG of receptors for component(s) involved in the reaction leading to bacterial death (2) Complement deviation by the IgG such that the complement system is more or less completely triggered off without leading to cell death

MATERIAL AND METHODS

Bacteria The *Salmonella typhimurium* m 395 MRO (chemotype Ra) chosen as a

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organism for this investigation has been described in detail earlier (14, 18, 8) The bacteria were kept on agar slants at 4° C and tested at intervals by phage pattern (18)

Immunization Synthetically grown, heat killed bacteria (0 2 mg dry wt) were injected intravenously into rabbits three times a week for nine weeks (7) The rabbits were bled from the carotid artery five days after the last injection The different rabbit sera used have the prefix ROL as described earlier (25) The sera were kept at −20° C and inactivated before use

Cattle serum Blood was collected under clean conditions at a slaughter house Serum was checked for sterility and kept at 90° C until used

Antibactericidal test One milliliter of an overnight culture of bacteria in 10 ml of nutrient broth was added to [illegible] ml of fresh broth and incubated for another 3 hours The bacteria were then washed twice and suspended in 0 03 M tris–HCl buffer (with 0 00015 M Ca^{++} and 0 0005 M Mg^{+}), pH 8 4 (called *tris buffer*) The bacterial concentration was estimated by turbidity in a Turner spectrophotometer at 620 nm The antibactericidal sample (rabbit serum or the material obtained after the pepsin digestion) was diluted in the same buffer To one part (0 15 ml) of rabbit serum an equal volume of cattle normal serum (diluted 1 2 in tris buffer) was added, followed by 2 parts of the bacterial suspension After 1 hour in a 37° C water bath the viable count was determined in duplicate on nutrient agar plates Plates with a colony number between 20 and 300 were counted, and the average of the two plates was determined The surviving fraction was calculated as N/N_0 where N_0 is the original concentration of bacteria and N the concentration at the test

Reproducibility of the antibactericidal tests The methodological error of the test was estimated by making 10 identical experiments on one day and also by performing the same test on 10 different days The bacterial suspension contained 4×10^5 bact /ml The rabbit antiserum ROL–35 was used in the final concentrations 1/256 and 1/512 At the concentration 1/256 the mean (x) of the $\log_{10}$ of the surviving fraction for concurrent tests was 0 52 standard deviation (s) = ± 0 10 for tests on different days x = 0 70 s = ± 0 18 At the concentration 1/512 concurrent tests showed x = 1 11 s = ± 0 15 and those on different days x = 1 10 and s = ± 0 24

Binding of rabbit antibodies to bacteria Equal volumes (1 5 ml) of a bacterial suspension (4×10^9/ml) and dilutions of rabbit immune serum ROL 10 in tris buffer were mixed After 20 min in a 37° C water bath the tubes were centrifuged and bacteria washed twice in tris buffer of the same volume as the supernatant fluid Samples (0 2 ml) of the suspension were withdrawn before the first centrifugation and after each resuspension To each sample, the same volume (0 2 ml) of cattle serum (diluted 1/2) was added, and the tubes were incubated in a 37° C water bath for 60 min Viable counts were determined as above Controls were performed with rabbit pre immune in place of immune serum and with both rabbit and calf sera replaced by buffer

Absorption of the rabbit immune serum with LPS LPS was extracted from dried cells of *Salmonella typhimurium* MRO by the method of *Galanos et al* (9) From a solution of 10 mg/ml LPS in dist water, 0 02 ml was added to 1 ml of antiserum ROL–35 and incubated at 37° C for one hour and at 4° C for another 3 hours After centrifugation at 20000 rpm for 15 min to deposit the precipitate, the supernatant was removed This sample was titrated in the antibactericidal test parallel to unabsorbed serum

The antibactericidal effect at different concentrations of bacteria Two fold dilutions of the antiserum ROL–35 from 1/16 to 1/256 in volumes of 0 15 ml were mixed with 0 15 ml cattle serum (1/2) and 0 3 ml of bacteria at concentrations of 4×10^3, 4×10^4, 4×10^5, 4×10^6, 4×10^7 and 4×10^8 bact /ml The surviving fractions at the different rabbit serum dilutions and bacterial concentrations were determined

Interruption of the bactericidal effect of cattle serum by rabbit antiserum Sixteen ml of a suspension containing 4×10 bact /ml were added to a mixture of [illegible] ml of cattle serum and 8 ml of tris buffer and incubated in a water bath at 37° C At different times samples of 0 5 ml were removed and added to 0 15 ml of a two fold serial dilution 1/1–1/128 of the antiserum ROL–35 and further incubated at 37° C for 1 hour, when viable counts were performed As a control, an ordinary antibactericidal test was done in which the rabbit antiserum and the normal cattle serum were first mixed, and the bacteria were added afterwards

Pepsin digestion The antiserum ROL–21 was pepsin digested by the method of *Nisonoff* (24) with slight modifications Saturated ammonium sulphate (room temperature) was added dropwise to an equal volume of serum The precipitate was washed once with saturated ammonium sulphate and then dissolved in distilled water as a two per cent protein solution This was dialysed at 4° C against 0 1 M sodium acetate buffer, pH 4 5 Pepsin (Worthington Biochemical Corp) dissolved in the same buffer was added, using 1 mg enzyme/50 mg protein Digestion was allowed to proceed at 37° C for 8 hours The pH was then adjusted to 8 3 by overnight dialysis against the tris buffer at 4° C The contents of the dialysis bag were precipitated once by adding an equal volume of saturated ammonium sulphate The precipitate was dissolved in distilled water and dialysed for 18 hours against the tris buffer at 4° C Two controls were included One treated as

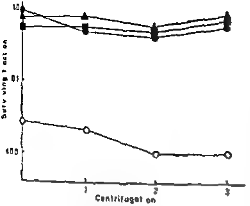

Fig 1 The bactericidal effect of cattle normal serum on bacteria incubated with rabbit antiserum and washed before the addition of the cattle serum O = rabbit pre immune serum Closed symbols = antiserum ROL–10 ● – undiluted serum ▲ – serum diluted 1/2 ■ – serum diluted 1/4 1 = after centrifugation, 2 – after first wash 3 – after second wash

above but without pepsin another dialysed against the tris buffer instead of the sodium acetate buffer pH 4 5 before the addition of pepsin

Protein concentrations were determined in a Beckman DB G spectrophotometer at 280 nm against a reference solution of human IgG (Kabi 1064) The three samples were simultaneously tested in two fold serial dilutions in antibactericidal tests using a bacterial concentration of 4×10^3 cells/ml The surviving fraction at each sample dilution was recorded and related to the protein concentration

RESULTS

Effect of washing on the antibactericidal effect After washing RO bacteria exposed to antiserum ROL–10 were still resistant to the bactericidal effect of cattle normal serum (Fig 1) The slight antibactericidal effect of rabbit pre-immune serum, however, vanished during the procedure Each centrifugation and resuspension decreased the viable count by about 25 per cent Therefore, the surviving fraction was calculated as the quotient of the viable count of ROL–10 treated to non treated bacteria subjected to the same number of centrifugations The surviving fraction in a control experiment without washing was 10 per cent at a final serum dilution of 1 512 (antibactericidal titre of ROL–10, E_{10} = 1 512)

Specificity of the rabbit anti RO serum One ml of antiserum ROL 35 was absorbed with 200 µg of RO LPS The precipitate was removed by centrifugation The supernatant

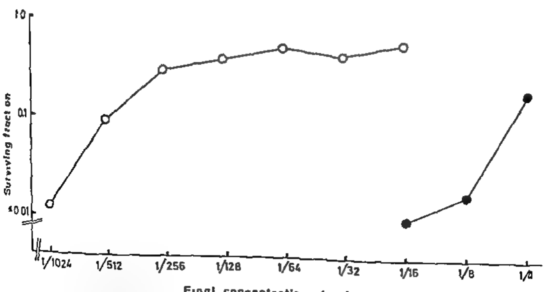

Fig 2 The antibactericidal effect of antiserum ROL–35 after absorption with 200 µg RO LPS per ml serum – ● Unabsorbed serum = ■

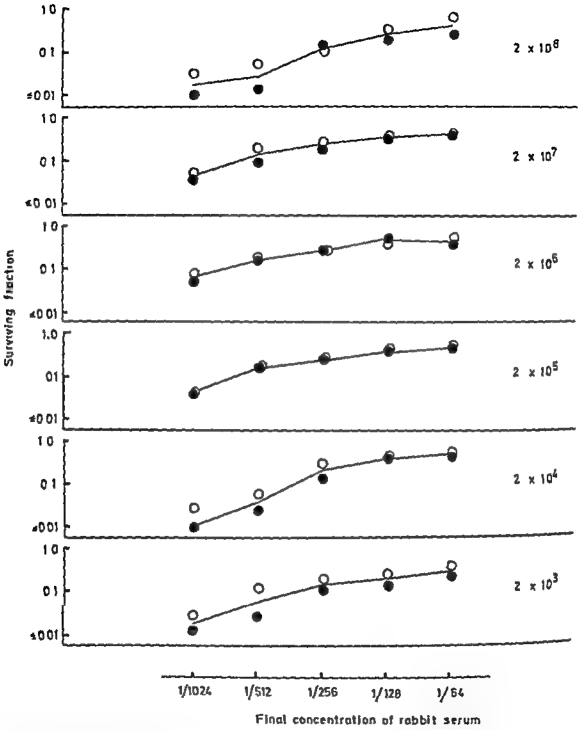

Fig 3 The antibactericidal effect of antiserum ROL–35 at different dilutions (1/64–1/1024) on different concentrations of bacteria ○ first series of experiments ● second series of experiments The lines are drawn through the geometric mean of the values obtained in the two series

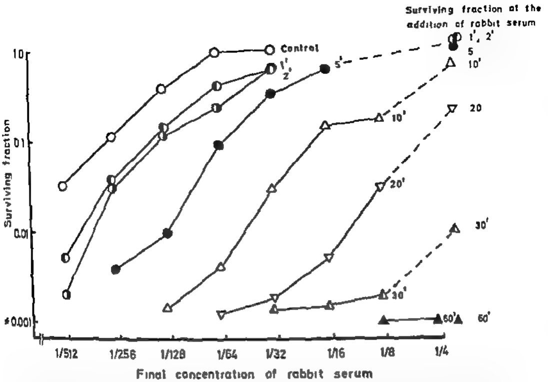

Fig 4 The antibactericidal effect of antiserum ROL-35 added at different times after the bacteria were mixed with cattle serum ◧ – 1 min ◑ = 2 min ● = 5 min △ – 10 min ▽ = [illegible] min △ – 30 min ▲ = 60 min The surviving fraction at the instant of addition of rabbit immune serum is recorded to the right in the figure In the control experiment rabbit and cattle serum were mixed before the addition of bacteria

was collected and its antibactericidal activity assayed at different dilutions and compared to that of unabsorbed serum (Fig 2) Ten per cent survival was obtained at antiserum concentrations between 1 4 and 1 8, whereas non absorbed serum showed E_{10} = 1 512

Relationship between bacterial concentration and antibactericidal effect The antibactericidal effect at different concentrations of bacteria was tested over a range of antiserum ROL-35 concentrations (Fig 3) At a final serum dilution of 1 64 the surviving fraction came within 0 28–0 49 for each concentration of bacteria, at dilution 1 1024 it was 0 01–0 06 For all bacterial concentrations, the antibactericidal titre (E_{10}) came within one dilution step of 1 512 At 200 bact /ml the same antibactericidal effect was observed Because of small numbers of colonies (0–10 per plate), these results are not shown in Fig 3

Kinetics of the antibactericidal effect of rabbit anti-RO serum When the antiserum ROL-35 was added at different times after mixing bacteria and cattle serum (Fig 4) the bactericidal process was interrupted and this occurred even as late as after 20 min Addition of the antiserum earlier saved more bacteria In every instance there was some bactericidal effect after addition of antiserum Higher concentrations of antiserum were more powerful in stopping the bactericidal effect

Effect of pepsin digestion Pepsin digestion decreased the antibactericidal effect of antiserum ROL-21 to such a degree that more than a 60 fold reduction of the antibactericidal activity occurred (Fig 5) To exclude non enzymatic effects on the reactions by pepsin, a control experiment with pepsin at pH 8 4, where it is inactive, was included To exclude effects on the rabbit serum by the

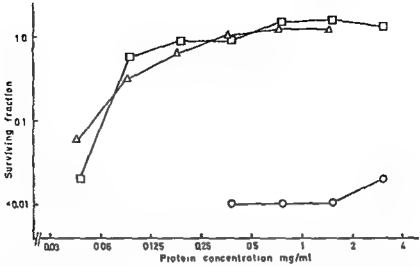

Fig 5 The antibactericidal effect of an antiserum ROL–21 after pepsin digestion at pH 4 5 (○) As a control pepsin was added at pH 8 5 (△) In another control experiment no pepsin was added (□) The surviving fraction is related to the protein concentrations of the treated samples

pH-changes another control was done at pH 4 5 without pepsin In both control experiments high antibactericidal activities persisted

DISCUSSION

The bactericidal effect of normal and immune sera on gram-negative bacteria is generally mediated by an activation of the complement system In the case of immune sera, antibodies against the cell wall O antigen seem to activate the complement system in a manner similar to immune haemolysis (22) In the case of normal serum other mechanisms also seem to exist *Pillemer et al* (27) thus claimed that properdin was involved in the bactericidal reaction of normal serum This substance could together with other serum factors, termed A and B, activate the complement system without specific antibody (3, 26) *Wedgewood* however proposed that in fact there was an additional need for antibody (31)

Analogous to the complement dependent killing of gram-negative bacteria by normal serum there is an activation of complement when LPS from such bacteria is added to normal serum This complement consumption by LPS requires a pre-incubation period and is rapidly lost upon dilution of the complement source (11) Since the bactericidal effect of cattle normal serum on S typhimurium 395 MRO was extinguished by the rabbit immune serum as late as after 20 min it seems likely that this type of complement activation by LPS is involved in the killing

The complement activation by LPS preferentially consumes each of the six terminal components C3, C5, C6, C7, C8 and C9 with minimal detectable depletion of the three earlier-acting components C1, C4 and C2 (11) It has been postulated that other serum factors are needed in addition to C3–C9, similar to those described for the properdin system (11, 13) It is not yet clear whether this alternate pathway for complement activation involves antibody and the early-acting complement components In earlier experiments (7) we found evidence for a thermolabile (56° C, 30 min) serum factor in human normal serum involved in the bactericidal reaction

Recent evidence suggests that the lipid moiety of endotoxic LPS can activate the complement system Thus the LPS from R-mutants, heptose defective and lacking almost all of the polysaccharide side chain, is almost as good a complement activator as the

LPS from the smooth strain (10) Such defective LPS exhibits most of the biological properties of the LPS from smooth bacteria (15 28) It is not known, however, whether the lipid moiety of the LPS is the exclusive target of the alternate pathway of complement activation

Incubation of *Veillonella alcalescens*, or LPS derived from it, with fresh guinea pig normal serum produces structural changes in the cell wall and LPS similar to those in red blood cell membranes undergoing immune haemolysis supporting the view that the lesions formed by complement in erythrocytes and in LPS are of the same nature (2) LPS forms much of the surface of the bacterial cell Since LPS can be annealed with cell wall phospholipid *in vitro*, it has been proposed that LPS and phospholipid may form a mixed continuous bilayer, which could be involved in the formation of the outer bacterial membrane (30) with the polysaccharide portion of LPS ■ the outermost structure Damage to this membrane appears essential for the bactericidal effect of serum Whether this damage results in cell death or merely allows access of potentially noxious serum substances such as lysozyme to a more deeply located target is not clear (12, 21)

Rabbit anti RO serum exerts its anti bactericidal activity by binding rather firmly to the bacteria as washing does not remove the activity (Fig 1) The rabbit antibodies bind to the cell wall LPS since absorbtion of the antiserum with LPS inhibits the reaction (Fig 2) High avidity of the anti RO antibodies is indicated since the LPS antibody complex is not significantly dissociated at pH 2 5 (*Normann & Cunningham*, unpublished)

One of the hypotheses suggested in the introduction implies that rabbit anti RO antibodies of the IgG class (25) inhibit bactericidal activity by blockade of receptors for any of the components involved in the killing Since pepsin treatment removed antibactericidal effect (Fig 5) blocking of receptors by the antigen binding part of the IgG molecule is not enough The apparent independence of the bacterial concentration (Fig 3) also contradicts steric blocking by the IgG molecule if it is assumed that an increased concentration of bacteria would raise the demand for blocking antibodies to protect target sites on the bacterial surface

Our results favour the other hypothesis The complement activity is deviated or scattered in such a way that a lethal lesion is not accomplished in the bacterial envelope Complement consumption by the rabbit anti-RO IgG, in the presence of RO bacteria has thus been demonstrated (to be published) The independence of the antibactericidal effect on the bacterial concentration may rather reflect a requirement for a critical *concentration* of bactericidal complement at the bacterial surface in order to kill the cell

Complement activation by anti RO IgG does not kill the bacteria but reduces the concentration of active complement, particularly at the bacterial surface, in such a way that the bactericidal process of cattle normal serum is interrupted (Fig 4) Since the latter process is slow and is rapidly lost upon dilution (11), the more rapid consumption by immune IgG diverts the complement for the slower bactericidal process The longer the bactericidal process has run, the higher concentration of anti RO serum is required to exhaust the bactericidal activity Similarly, higher concentrations of cattle serum require higher concentrations of rabbit antiserum to inhibit the bactericidal reaction (unpublished) Analogous results appeared in a study of the Neisser-Wechsberg phenomenon by *Muschel et al* (20), where the prozone inhibition of the bactericidal effect of rabbit immune serum against *S typhi* O 901 was overcome by addition of extra complement

Conglutinin is present in bovine serum and immunoconglutinin arises in sera from several species after immunization Both of these react with activated C3 (16, 19) The C3 inactivator (conglutinogen activating factor = KAF) is well characterized and present in many animal sera including human sera (17) It attacks C3 b and cell bound C3, but not active C3 (1) These serum factors may be involved in the bactericidal

as secondary phenomena However, since the inhibition of the bactericidal process is immunologically specific and due to IgG, they probably play no major part in the antibactericidal reaction

The molecular mechanism of the extinction of the bactericidal effect of serum is still obscure If complement activation by LPS via the alternate pathway is essential for killing, the presence of large quantities of specific IgG antibodies may reduce the concentration of one or more later complement factors (C3–C9) below the crucial level for the slower activation by the deeper (lipid?) moiety The complement activation by IgG antibodies alone, however, does not cause fatal lesion

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CAFFEINE SENSITIVE RECOVERY OF MITOMYCIN C TREATED MOUSE P-388 CELLS

JON ØRSTAVIK

Mouse P 388 cells were grown in suspension culture and given a short treatment with 0 5 μg/ml of mitomycin C The effects of the pyrimidine analogue caffeine on cell growth DNA synthesis and DNA degradation in such mitomycin C treated cultures was then investigated Post treatment incubation with 5 10 $^{-4}$ M of caffe ne strongly potentiated the cytotoxic effect of mitomycin C Exposure to the antibiotic for 1 hour inhib ted cell growth only during the first day after treatment In contrast when these cells were incubated in medium containing caffeine cell multiplicat on was suppressed during the whole experi mental period Low concentrations of caffeine prevented mitomycin C induced inhibition of the incorporation of thymidine into DNA Maximum stimulation of ^{3}H TdR incorporation in mitomycin C treated cells was found in cultures supplemented with 5 10 $^{-4}$ M of caffeine Cells labelled with ^{3}H TdR prior to treatment with mitomycin C did not release significant amounts of rad oactivity to the culture medium Post treatment incubation with caffeine did not change this finding

The cytotoxicity of mitomycin C is believed to depend on its ability to alkylate cell DNA thereby blocking DNA replication (*Szybalski and Iyer* 1967) In bacteria, mitomycin C resistance is related to their ability to perform DNA repair (e g *Boyce and Howard Flanders* 1964) but little is known about similar phenomena in mammalian cells In a previous report data were presented indicating that mouse P 388 cells recover after short trea ment with the antibiotic (*Orstavik* 1972 a suggesting the existence of a repair mechan ism in these cells

In the present study the effect of the pyrimidine analogue caffeine on the recovery of mitomycin C treated mouse cells in tissue culture has been investigated Growth of mammalian cells after exposure to various inhibitors of DNA synthesis is highly sensitive to caffeine (*Rauth* 1967, *Gaudin and Yielding* 1969, *Rauth et al* 1970) It has been suggested that caffeine might act by sup pressing repair of DNA damage, but con clusive evidence to this effect has not been presented It has also been suggested that caffeine might potentiate the DNA damage (*Domon and Rauth* 1969, *Domon et al* 1970), or inhibit as yet unknown recovery mechanisms other than DNA repair (*Cleaver* 1967)

The data in the present report show that recovery of cell growth after short treatment with mitomycin C was inhibited by 5 10 $^{-4}$ M of caffeine The same concentration of caf feine stimulated the incorporation of ^{3}H thymidine into DNA of such mitomycin C treated cells

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MATERIALS AND METHODS

Cell Cultures

P 388 mouse cells (*Dawe and Potter* 1957) were grown in suspension culture on Eagle's minimum essential medium supplemented with 10 per cent foetal bovine serum as described previously (*Ørstavik* 1972 a).

Treatment with Mitomycin C and Caffeine

The cells were incubated at 37° C in medium containing 0.5 μg/ml of mitomycin C for the time given for each experiment. The cells were then harvested by centrifugation, washed in growth medium and re-incubated in medium without antibiotic. Control cultures were supplied with a corresponding volume of distilled water and given the same treatment.

Caffeine was added to the medium after treatment of the cells with mitomycin C. Cultures grown for more than 1 day were diluted daily with fresh medium containing the same concentration of caffeine.

Cell Growth

Cell multiplication was followed by counting the cells in a haemocytometer.

Fractionation of Cells

The culture medium was removed by centrifugation and the cells were washed with cold PBS*. The cells were then extracted twice with 5 per cent (w/v) TCA for 20 minutes at 0°C and subsequently for 20 minutes at 90° C. Acid insoluble cell material was finally dissolved in 1 N KOH.

Synthesis and Degradation of DNA

The rate of DNA synthesis was determined by measuring the incorporation of radioactive thymidine into the hot TCA soluble extracts. Degradation of DNA was measured as release of radioactivity to the culture medium from cells which had been prelabelled with ^{3}H thymidine as described previously (*Ørstavik* 1972 b).

Measurements of Radioactivity

Radioactivity was measured in a Packard Tri-Carb model 3365 liquid scintillation counter. Samples of 25 μl were counted in 10 ml toluene containing 4 g/l of PPO, 0.05 g/l of POPOP and 15 per cent of etyleneglycol monomethyl ether.

* Abbreviations: PBS, phosphate buffered saline, 0.15 M, pH 7.2; TCA, trichloro-acetic acid; MC, mitomycin C; ^{3}H TdR, thymidine 6 T(n); PPO, 2.5-diphenyloxazole; POPOP, 1.4-bis-2 (4-methyl-5-phenyloxazolyl) benzene.

Chemicals

Mitomycin C was obtained from Kyowa Hakko Kogyo Co., Tokyo. Aqueous stock solution (1 mg/ml) was prepared the day before use. Caffeine (anhydrous, Calbiochem) was dissolved in water (100 mM) and sterilized by filtration. Thymidine [illegible] T(n), specific activity 3–5 Ci/mmol (The Radiochemical Centre, Amersham) was kept as sterile aqueous solution (100 μCi/ml) and renewed every second month. All solutions were kept at 4° C.

RESULTS

Effect of Caffeine on Recovery of Cell growth after Treatment with Mitomycin C

From the results in Fig. 1 it can be seen that 0.5 mM of caffeine did not affect the growth rate of P-388 cells in suspension culture. In agreement with our previous results (*Ørstavik* 1972 a) exposure to 0.5 μg/ml of mitomycin C for 1 hour inhibited cell multiplication during the first day after treatment. Thereafter the cells resumed a normal growth rate. In contrast, if this treatment

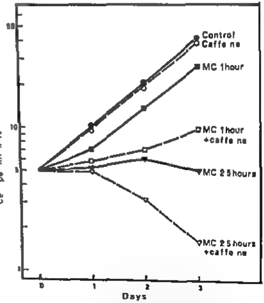

Fig. 1. Effect of caffeine on cell growth after treatment with mitomycin C. Control cells and cells treated with 0.5 μg/ml of mitomycin C for 1 and 2.5 hours were grown for 3 days in pure medium and in medium supplemented with 5 · 10⁻⁴ M of caffeine. Mean cell counts from duplicate cultures have been recorded.

with mitomycin C were followed by incubating the cells in medium containing $5 \cdot 10^{-4}$ M of caffeine, cell multiplication would be strongly suppressed during the whole experimental period

Cells which had been treated with 0.5 μg/ml of mitomycin C for 2.5 hours did not recover from the growth inhibition induced by the antibiotic (Fig 1) Obviously, post treatment incubation with caffeine again potentiated the cytotoxic effect of the mitomycin C treatment

Caffeine Induced Stimulation of ³H-Thymidine Incorporation in Mitomycin C Treated Cells

In a previous report (*Ørstavik* 1972 b) it was shown that mitomycin C rapidly suppressed DNA synthesis in P-388 cells The results in Fig 2 confirmed this finding Thus, exposure to 0.5 μg/ml of the antibiotic for 2.5 hours followed by growth in pure medium for 1 hour caused nearly 50 per cent inhibition of thymidine incorporation into the hot TCA soluble cell fraction It further appears (Fig 2 a) that the mitomycin C induced inhibition of DNA synthesis was prevented by the presense of low concentrations of caffeine in the medium The effect of caffeine increased with increasing concentrations up to about $5 \cdot 10^{-4}$ M and was specific for mitomycin C treated cells since it was found that caffeine did not affect the incorporation of thymidine in untreated control cells Measurements of the radioactivity in the cold TCA soluble cell extracts (Fig 2 b) showed that the stimulatory effect of caffeine on DNA synthesis in mitomycin C treated cells was not due to enhanced cellular uptake of labelled thymidine from the medium

In cultures where the concentration of caffeine exceeded $5 \cdot 10^{-4}$ M, the incorporation

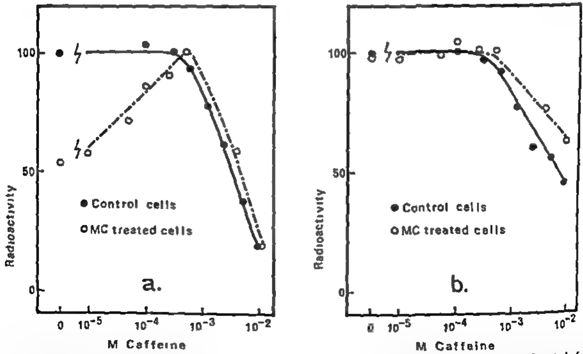

Fig 2 Effect of caffeine on incorporation of ³H thymidine Control cells and cells treated with 0.5 μg/ml of mitomycin C for 2.5 hours were incubated in medium containing various concentrations of caffeine After 1 hour the cells were labelled with ³H TdR for 15 minutes a Radioactivity in hot TCA soluble cell extracts b Radioactivity in cold TCA soluble cell extracts Mean values from duplicate cultures have been recorded as per cent of the corresponding values from control cells grown in caffeine-free medium.

of ^{3}H TdR in DNA was strongly suppressed (Fig 2 a) However, the uptake of radioactivity in the cold acid soluble cell fraction was also strongly inhibited (Fig 2 b) These effects were similar in untreated and in mitomycin C treated cells

Influence of Caffeine on DNA Degradation in Mitomycin C Treated Cells

The results obtained in a previous study (*Orstavik* 1972 b) indicated that degradation of DNA was not an early effect of mitomycin C treatment in P-388 cells The data presented in Table 1 confirm this ^{3}H-TdR labelled cells exposed to 0 5 μg/ml of mitomycin C for 2 5 hours did not release radioactivity to the culture medium during the first hour after treatment if they were grown in pure medium Similar results were obtained if such mitomycin C treated cells were grown in medium containing 5 10^{-4} M of caffeine This indicate that post treatment incubation with caffeine did not affect degradation of DNA in mitomycin C treated cells The results also show that the same concentration of caffeine did not induce DNA breakdown in untreated P-388 cells It was calculated that more than 98 per cent of

TABLE 1 *Effect of Mitomycin C and of Post Treatment Incubation with Caffeine on Degradation of DNA*

Mitomycin C (µg/ml)	Caffeine (M)	Radioactivity in culture medium (cpm/ml) Minutes of incubation 15	45	75
0 (control)	0	72	--	28
0 (control)	5 10^{-4}	76	[illegible]	16
0 5	0	40	16	8
0 5	5 10^{-4}	46	50	38

Cells labelled with ^{3}H thymidine were grown for 2 5 hours in pure medium (control) or in medium containing 0 5 μg/ml of mitomycin C washed and incubated in medium with or without [illegible] 10^{-4} M of caffeine Samples were removed at the intervals indicated and the radioactivity in the culture medium was measured by scintillation counting Mean values from duplicate cultures have been presented

the total radioactivity remained in the hot TCA soluble cell fraction in all the cultures

DISCUSSION

Increased cytotoxicity of short treatment with mitomycin C when the post treatment incubation medium contained caffeine (Fig 1) is in agreement with a previous study on mitomycin C treated mouse L cells (*Rauth et al* 1970) The finding might implicate that caffeine either potentiated the mitomycin C induced cell damage, or that the cells contained a caffeine sensitive mechanism for circumvention or repair of this damage

Since mitomycin C rapidly suppresses DNA synthesis in the P 388 cells (*Orstavik* 1972 b) and the cytotoxicity of the antibiotic is believed to depend on alkylation of DNA (*Szybalski and Iyer* 1967), it might be suspected that caffeine acted by increasing the inhibition of DNA synthesis, or by interfering with a process for repair of alkylated DNA It was therefore a surprising observation that caffeine did not further suppress, but stimulated incorporation of thymidine in the mitomycin C treated cells (Fig 2 a) Since caffeine did not stimulate thymidine incorporation in control cells it does not seem likely that semiconservative DNA replication was increased A possible explanation might be that the mitomycin C treated cells carried out part of their DNA synthesis by a different mechanism This caffeine stimulated form of DNA synthesis could be associated with DNA repair, and possibly be similar to the DNA 'repair replication' found in mammalian cells treated with other DNA inhibitors (e g *Cleaver and Painter* 1968 *Roberts et al* 1971)

It might seem contradictory that caffeine should stimulate repair replication and at the same time suppress recovery of cell growth Repair replication is however, but one of several steps in the process leading to the reconstitution of functional DNA (review *Strauss* 1968) Therefore, an increased level of repair replication is not necessarily incompatible with inefficient DNA repair One

possible explanation for the present findings might be that caffeine interfered with a DNA repair process by blocking the final ligase step Thereby the repair replication might proceed along more extended regions of the damaged DNA strand than in a normal repair process *Cleaver and Thomas* (1969) showed that caffeine prevented UV irradiated chinese hamster cells, but not unirradiated controls, from transforming newly synthesized, short DNA strands into extended molecules Their finding is also compatible with the view that caffeine may inhibit a DNA repair process at the final ligase step

DNA synthesis as measured in the present study does not distinguish between semiconservative DNA replication and repair replication in the mitomycin C treated cells Conclusions regarding the existence of the latter process—as part of a repair system responsible for the caffeine sensitive recovery of cell growth—should therefore not be drawn DNA repair replication in mitomycin C treated P-388 cells is currently being investigated

Since post treatment incubation with caffeine did not alter the finding that short treatment with mitomycin C does not cause detectable degradation of DNA (Table 1, *Orstavik* 1972 b) it might appear that excision and depolymerization of DNA lesions was not part of the mechanism leading to recovery of cell growth However, since only minor segments of DNA are usually reconstituted in a DNA repair process (*Roberts et al* 1971) and labelled DNA degradation products may be reutilized (*Cleaver* 1967), a limited DNA degradation connected with DNA repair might proceed undetected in the present cultures

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IMMUNOELECTROPHORETIC PATTERNS OF EXTRACTS FROM *ESCHERICHIA COLI* O ANTIGEN TEST STRAINS O1 TO O157 EXAMINATIONS IN HOMOLOGOUS OK SERA

Further Comments on the Classification of Escherichia *K Antigens*

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All *Escherichia coli* O antigen test strains were examined by immunoelectrophoresis in homologous O and OK sera The immunoelectropherograms could be subdivided into few well defined groups according to the movement of the O antigen and of the thermostable polysaccharide K antigen if present The outcome of the examination confirmed a previous suggestion that only a limited number of test strains have a special polysaccharide K antigen The previously indicated relationship between pathogenicity and immunoelectrophoretic pattern was emphasized

Recently we published a paper demonstrating that simple water extracts of the *Escherichia coli* antigenic test strains by immunoelectrophoresis (IE) could be grouped in a few well defined categories (*11*) It was suggested that some correlation existed between these IE patterns and pathogenicity For that first publication extracts of O antigenic test strains O1 to O150 were examined in homologous O sera and K antigenic test strains K1 to K91 were examined in homologous O and OK sera but OK sera corresponding to all O test strains O1 to O150 were not available

For the present communication, further examinations of extracts of O1 to O150 and of some recently added O groups O151 to O153 and O157, were carried out in the homologous OK sera For a more general introductory discussion of the development of the K antigen concept and of our present ideas about the status of *E coli* K antigens the reader is referred to the previous papers in this series (*10 11, 12, 13*)

METHODS

Medium The D5 medium described by *Schlecht & Westphal* (*15*) was used with 0 05 per cent

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the O test strains have been removed and not substituted, so the total number of strains examined was 150

Extraction The bacteria were cultivated on the modified D5 medium at 37° C for 20 hours The culture from four 14 cm diameter plates was scraped off and suspended in 10 ml saline giving about 0 3 g wet weight of bacteria per ml This suspension was heated to 60° C for 20 minutes in a waterbath followed by centrifugation at 8000 *g* for 15 minutes The supernatant was labelled extract 60° 20 and part of it was heated to 100° C for 1 hour and labelled 100° The extracts could be kept for several months at 4° C

Sera Rabbit O and OK sera were used some of which were routine sera from the International Escherichia Centre (WHO) and some were OK sera produced for this examination corresponding to the O antigen test strains with no numbered K antigen The sera were produced according to traditional procedures (4)

The immunoelectrophoretic technique was that of *Scheidegger* (14) using the equipment manufactured by LKB of Sweden Three glass slides were coated with 9 ml 1 5 per cent agar Noble in veronal buffer pH 8 6 ionic strength 0 05 μ Wells and troughs were cut with LKB device The agar from the wells was sucked off and the wells filled with 1 1 5 μl antigen solution Veronal buffer, pH 8 6 ionic strength 0 05 μ was used in the electrophoretic chamber The electrophoretic separation was performed at a voltage of 7 V/cm for 2 hours The agar in the trough was lifted out and the trough filled with about 150 μl undiluted antiserum The slides were left in a moist chamber overnight Before staining the precipitation patterns were recorded graphically by drawing The slides were washed for 2 days in saline and on the 3rd day washed in distilled water before staining with Amidoschwarz

RESULTS

The main results of the immunoelectrophoretic examination of extracts of all *E coli* antigen test strains in their homologous O and OK sera are recorded in Fig 1 It is apparent that all IE patterns can be grouped in the same few main groups described in an earlier paper on the *E coli* antigen test strains and also that the prediction made in that paper on the expected outcome of the examination of all O test strains in the homologous OK sera was fulfilled (11)

The IE patterns given by O antigens in homologous O sera of the first 150 *E coli* groups have already been described in the earlier publication The general outcome of that study has, as expected, not been changed by the additional examination in homologous OK sera The O precipition arcs of coli O antigens are practically always located close to the application basin but can be placed into two main groups according to their position on either the cathodic or the anodic side of the basin The two groups have been called groups 1 and 2 both in the present and in the previous communication That this line is caused by precipitation of the O antigen can be confirmed by the reaction of identity if extracts from different strains with the same O but with different K antigens are compared It is not uncommon to find the O precipitation arc as two or three parallel lines, reports are available according to which similar observations frequently have been made when the Ouchterlony technique were used for the examinations It is not feasible to publish all the actual immunoelectropherograms and therefore the results have been recorded in schematic form in Fig 1 It is characteristic that different extracts of the same strain or serotype examined at different time give the same pattern As expected, different sera can give results which vary quantitatively i e a precipitation line of one rabbit may be weaker than that of another rabbit, but the position of the line will not differ Some few extracts will give O precipitation arcs that cannot easily be put either into groups 1 or group 2, the O antigen does not seem to move very far from the application basin Such cases are O39 O79, O80 and O81

The small extra anodic O precipitation arc previously found in extracts of some strains of IE group 1 was also found in the additional strains examined in this investigation We suggested in our previous publication that the presence of this anodic O precipitation line was correlated with the presence of a polysaccharide K antigen However, it should be emphasized that this small line was not found in every case where we expected to find it and in some cases it was

ANALYSIS OF ESCHERICHIA COLI O ANTIGEN TEST STRAINS IN IMMUNOELECTROPHORESIS IN HOMOLOGOUS O AND OK SERA

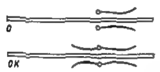
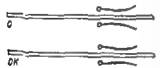
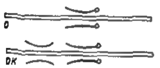
Group		Pattern	Strains
1A	a		03 K2ab 04 K3 05 K4 06 K2ac 011 K10 013 K11 015 K14 017 K16 020 K17 021 K20 023 K18 025 K19 044 K74 049 K? 075 K? 077 K? 0103 K? 0107 K? 0117 K?
	b		01 K1 02 K1 07 K1 010 K5 012 K5 016 K1 045 K?
1B	a		08 K8 09 K9
	b		018 K76 019 K⁻ 026 K60 034 K? 035 K? 036 K? 039 K? 040 K? 043 K? 050 K? 051 K? 052 K? 055:K59 060 K? 062 K? 063 K? 066 K? 068 K? 070 K? 071 K? 073 K? 078 K80 085 K? 086 K⁻ 088 K? 089 K? 090 K? 092 K? 095 K? 097 K? 099 K? 0101 K? 0102:K? 0106 K? 0109 K? 0111 K58 0114 K90 0118 K? 0119 K69 0123 K? 0125 K70 0126 K71 0127 K63 0128 K67 0129 K? 0132 K? 0135 K? 0142 K86 0145 K? 0148 K? 0151 K? 0153 K? 0157 K?
2	a		014 K7 022 K13 081 K? 0116 K? 0120 K? 0150 K93
	b		024 K⁻ 027 K? 028ab K? 028ac K73 029 K? 030 K? 032 K? 033 K? 037 K? 038 K? 041 K? 042 K? 046 K? 048 K? 053 K? 054 K? 056 K? 057 K? 058 K? 059 K? 061 K? 064 K? 065 K? 069 K? 074 K? 076 K? 079 K? 080 K? 082 K? 083 K? 084 K? 087 K? 091 K? 093 K? 096 K? 098 K? 0100 K? 0104 K? 0105 K? 0108 K? 0110 K? 0112ab K68 0112ac K66 0113 K75 0115 K? 0121 K? 0124 K72 0130 K? 0131 K? 0133 K? 0134:K? 0136 K78 0137 K79 0138 K81 0139 K82 0140 K7 0141 K85 0143 K? 0144 K? 0146 K? 0147 K89 0149 K91 0152 K?

Fig 1 Immunoelectropherogram In the trough homologous O or OK *antiserum* In the well above trough 60° 20' extract In the well below trough 100° extract The right column contains the O K serogroups of all *E coli* O antigen test strains K? means that the K antigen has not been examined Most of such strains have probably no special independent polysaccharide K antigen (see text)

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only weakly developed. Probably this diversity can be explained by the lability of the forces which cause some of the neutral O lipopolysaccharide molecules to move towards the anode.

In the first paper in this series it was shown that the fact that some O lipopolysaccharides (IE group 2) move towards the anode very probably is determined by their content of acidic components, most often hexuronic acid, N-acetylneuraminic acid or phosphate groups (11).

Group 1Aa contains many of the *Escherichia* strains with low O numbers which are equipped with a special polysaccharide capsule detected by the anodic thermoresistant precipitation arc found in IE using OK sera. As stressed previously, many of these capsulated test strains are at the same time representatives of the O groups found most often in the normal colon and those found in extra-intestinal disease (9).

Group 1Ab has also been discussed before and it shall only be mentioned here that the strains containing K1 antigen do contain a thermostable anodic substance which by staining can be detected at the position where we would expect a K antigen. It can be difficult to produce agglutinating and precipitating K1 antisera, a difficulty which is caused by some sort of form variation in the K1 antigen and probably also by the rather poor immunogenicity of this antigen or part of it. When K1 antisera which give reasonable K agglutination titres are used for IE, the non-boiled extract shows a precipitation arc at the position of an anodic K antigen (11). No precipitation arc for the boiled extract is found. As mentioned before, it is highly probable that these K1 strains contain a polysaccharide K antigen, but the immunogenic properties, at least in rabbits, seem to be different from those normally found. Neuraminic acid is probably the acidic component or one of the acidic components in this polysaccharide.

The strains in group 1Ba also contain cathodic O antigens and an anodic thermos[illegible]le K antigen. The only difference between these IE patterns and those in groups 1Aa and 1Ab is that the small anodic O antigenic spur is not seen, perhaps because it would be covered by the K antigen arc which is anodic but which, as a rule, does not move very far from the application well. It is a well established fact that the O8, O9, O101 strains with K antigens of the A type differ from the remaining K antigen containing *Escherichia* strains by the increased thermostability of the A type antigen. These strains grow with more mucoid colonies and the inagglutinability in O serum is only removed by heating to 120° C. In IE they are characterized by weak O precipitation arcs. If the usual extracts are heated to 120° C they will give more distinct O precipitation arcs. If K minus variants are used for extract production, quite typical cathodic precipitations will be found. These facts all indicate some sort of binding between the O lipopolysaccharide molecule and the polysaccharide K molecule which is abolished by heating.

In this connection it could be mentioned that extracts of a number of K minus mutants from the polysaccharide K containing strains of IE group 1Aa have been examined with a view to their IE pattern. They show, as expected, no K line and a cathodic O line without the small extra anodic O line.

In the previous paper it was suggested that most of the O test strans listed as IE groups 1Bb and 2 would not have an independent K polysaccharide. This suggestion is nicely confirmed by the present examination of all O test strains in their homologous O sera.

Most of the O antigen test strains do not have special polysaccharide K antigens.

Regarding the question of a relationship between special serogroups and certain pathological conditions, the present examination could only amplify the main results obtained in the earlier one.

The OK groups from human infantile diarrhoea fall into IE group 1Bb; these strains have no special K polysaccharide antigens.

The strains representing the O groups found most frequently in the normal human

colon and in extra intestinal disease are characterized by special K polysaccharide antigens and are found in IE groups 1Aa, 1Ab, 1Ba and 2a

The O groups found in strains associated with dysentery like disease all belong in IE group 2 and have anodic O lipopolysaccharides None of these strains have polysaccharide capsules

The results previously published (11) showed that most of the OK groups which are commonly found in diarrhoea diseases in young pigs belong in IE group 2, in contrast to the OK groups from human infantile diarrhoea which most often belong in IE group 1Bb

DISCUSSION

In this paper we shall not repeat the discussion presented in our previous reports on related topics (10 11, 12, 13)

Kauffmann, who introduced modern serological methods in the *Escherichia* group (4, 5 6), has recently written a short comment (7) to our first paper on IE patterns of *E coli* antigens (11) We shall not try to make any extended reply to *Kauffmann*'s comments several of which are completely agreeable to us However, a few facts should perhaps be pointed out *Kauffmann* writes that our finding of many strains with K(B) antigens in which special K antigens are absent does not mean that these strains have no special B antigens, but only that the IE method has failed to detect them We should like however, to point out that the absence of a failing K precipitation line in most of the strains with B antigen is not the only reason suggesting the absence of an independent K polysaccharide in most of the B antigen containing strains This conception is also supported by *Jann et al* (2, 3) who showed that at least in some B containing strains it was not possible chemically to differentiate between the O and the B specificity

Based on the findings in our paper, *Kauffmann* proposes the word 'electrotype' for the IE groups, but we find the term immunoelectrophoretic pattern, or immunoelectropherogram to be more informative

We have already proposed some changes in the present nomenclature of the surface antigens (9), namely that new K numbers should only be given to surface antigens which chemically and serologically can be differentiated from the O antigen lipopolysaccharide A logical consequence would be that the K term should no longer be applied to cases where such independent K antigen cannot be detected, e g instead of using the terms O111 K60 H2 and O55 K59 H6 to use O111 H2 and O55 H6, in fact, to abolish many established B antigen numbers together with the term B antigen Up to now it has been the custom to leave out an unknown K number or to put in a K? in the serotype formula In Fig 1 there are many such K? According to our present knowledge and especially to the IE results presented here, many of these strains do not contain special K antigens and it would be correct to delete the K?, e g the test strain for O group 35 (E77a), which is now termed O35 K? H10, should be termed O35 H10 In this context it should be emphasized that some day it might well happen that a true independent K polysaccharide would be found in another strain with O group 35 If this polysaccharide antigen was not already known in connection with another O antigen, it should have its own new K number

We have deliberately not changed the nomenclature in Fig 1 because we think that both the nomenclatural changes discussed above and further necessary changes should first be discussed and preferably agreed upon in the relevant international committees

In our first paper on IE of *E coli* strains we were impressed by the neat manner in which many of the strains isolated from pathological conditions could be fitted into different IE groups The present project included a further examination of a large number of OK sera and the previously advocated assumption that only rather few of the test strains had special polysaccharide K antigens

fimbriated, whereas electron micrographs of preparations made from non-spreading, non-corroding colonies (N type) most often contain no fimbriae at all. A low-frequent, bidirectional covariation of colony and fimbriation type was described. A similar correlation has been found in the species *M. nonliquefaciens* and *M. kingii* (2, 3, 6). Also, in the same three species, correlations between fimbriation, the ability to form a surface pellicle in fluid medium (2, 3, 6), twitching motility (8) and competence in genetic transformation (4) have been described.

It was reported by *Pedersen* (16, 17) that primary cultures on bovine blood agar of *M. bovis* from natural outbreaks of infectious bovine keratoconjunctivitis consisted of flat, rough colonies, and that often smaller, convex and smooth dissociants occurred in subculture. It was the aim of the present study to relate these colony appearances to those found by *Bøvre & Frøholm* (3) under different cultural conditions and to examine, by experimental inoculation with controlled fimbriation variants, whether there is a connection between fimbriation of *M. bovis* and its ability to colonize the bovine conjunctiva and to elicit keratoconjunctivitis.

MATERIALS AND METHODS

Bacterial Strains

Ten cell lines* of *M. bovis* were examined (Table 1). They originated from the strains 3, 4, 5 and 9 previously described by *Pedersen* (16). All cell lines have been included in studies on colony morphology, fimbriation and competence in genetic transformation by *Bøvre & Frøholm* (refs. 3 and 4). Some of them have also been examined for twitching motility (8).

Experimental Animals

Calves of the Red Danish Milkbreed and the Black and White Danish Cattle were used. The age of the calves varied from 2 to 5 months. The animals were kept indoors in individual pens. They were all purchased from herds in which allegedly infectious keratoconjunctivitis had never occurred.

* The term cell lines denotes clones kept in sub-[illegible] on blood agar for different lengths [illegible] and ref. 3.

Infection Procedure and Bacteriological Examination

Before infection experiments the conjunctival flora was controlled by swabbing and culture daily for 3 days, in particular with respect to the absence of *M. bovis*. In each calf one of the eyes was selectively irradiated twice with ultraviolet light, for 10 minutes at a distance of 60 cm the day before and again immediately prior to the inoculation (10). Both eyes were inoculated by installation into the lower conjunctival sac with the same bacterial culture. The inocula consisted of 0.2 ml of overnight cultures in horse serum broth (16). The number of colony forming units inoculated in each case is given in Table 1. Conjunctival swab cultures were thereafter made daily, beginning 24 hr after inoculation, until *M. bovis* could no longer be recovered. Primary cultures were examined on bovine blood agar medium (16), and the cultures were studied further both on this medium and on human blood agar plates according to the standard procedures used by *Bøvre & Frøholm* (3). The technique of electron microscopical examination for fimbriae was as previously described (3, see also legend to Fig. 5).

RESULTS

The typical characteristics of 1) the large, flat and rough and 2) the most often smaller convex and smooth colony type of *M. bovis* on bovine blood agar (16) were reproduced on the same medium when incubation was performed in a humid atmosphere, both at 37° C and 33° C. The wrinkled granular surface of the flat colony type was very distinct after 24 hr of incubation (Fig. 1). Macroscopically visible spreading zones were not observed. Upon further incubation, however, the flat colonies became more even and glistening (Fig. 2). At the same time, an increasing proportion of the progeny colonies on bovine blood agar medium were found to be variants of the convex (smooth) type (Figs. 3 and 4). It was found that the large and flat (rough) colonies left a corroded area in the bovine blood agar (Figs. 2 and 3), regardless of temperature and humidity, whereas the most often smaller and convex (smooth) colony type usually did not. When grown in statically incubated horse serum broth (16) at 37° C, the flat, corroding type formed a surface pellicle. The fluid medium

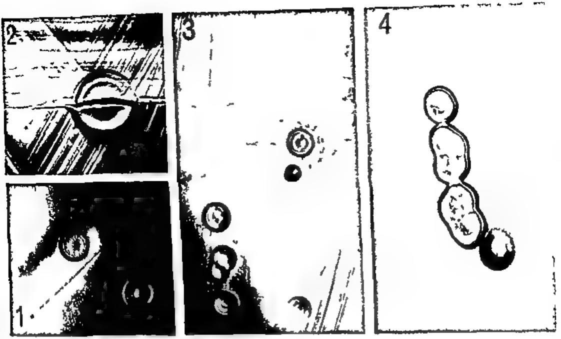

Fig 1 *M bovis* 9, bovine blood agar culture incubated for 24 hr at 37° C Typical flat colonies with rough wrinkled surface and elevated border (6 ×)

Fig 2 *M bovis* 9 flat colony in primary culture from eye of diseased calf cultural conditions as above for 40 hr The upper half of the colony has been removed to show corrosion (5 3 ×)

Fig 3 As Fig 1, showing one smaller, convex (smooth) colony together with several flat ones In the lower right quadrant, the corrosion left after removing a flat colony is seen In the middle of the picture the agar surface is uneven due to the condensation of water vapour in microdroplets (6 ×)

Fig 4 As Fig 1 showing several flat colonies and a single convex (smooth possibly mixed) colony The size of the latter as compared with the flat ones is greater than in Fig 3 (6 2 ×) (photo L Eriksen)

had a low turbidity In the bottom of the tubes there were varying amounts of sediment which formed coarse granular suspensions when the tubes were shaken Cultures in fluid medium from the convex, non corroding colony type grew as almost homogenous suspensions with no trace of a surface pellicle (see refs 3 and 4 for SC and N colony type characteristics)

Already in the first transfer from bovine to human blood agar, the progeny of flat, corroding colonies most often appeared as small, corroding and slightly spreading (SC) colonies (Fig 9 in ref 3) The flat, non-spreading colonies distinctly larger than the non corroding type found on bovine agar were not observed on the human blood agar medium although a slight central flattening could sometimes be seen in SC colonies growing separately and thus attaining larger dimensions than usual As the flat, corroding parent colonies on bovine blood agar aged, a varying proportion of the progeny on human blood agar became of the non corroding (N) variant type An enrichment of non corroding variant cells within aged corroding colonies has been observed also after long series of single colony transfers on human blood agar (3) The general statement can therefore be made that the large, flat and rough colony type described by *Pedersen* (16) corresponds to the SC type of colony when transferred to the cultural conditions used by *Bovre* & *Froholm* (3)

However, after maintenance of the SC cell lines for a longer period in passages on h

TABLE 1 *Experimental Conjunctival Infection of Calves with Fimbriation Variants of* Moraxella bovis*

Strain	Colony type§ – cell line†	Degree of fimbriation*	Inoculum (colony forming units)	Reisolation of *M bovis* (days after inoculation)		Disease observed after inoculation	
				UV irradiated eye	Non irradiated eye	UV-irradiated eye	Non irradiated eye
3	N a	—	$1\ 2\times10^{7}$	0	0	0	0
	SC–a	+ +	$6\ 8\times10^{6}$	+ (7)	0	0	0
4	N–b	—	$1\ 1\times10^{3}$	0	0	0	0
	SC–a	+ +	$4\ 0\times10^{3}$	+ (15)	0	Conjunctivitis	0
5	N a	—	$1\ 9\times10^{8}$	0	0	0	0
	N–c	(—)	[illegible] 2×10^{4}	0	0	0	0
	SC–a	+ +	$7\ 4\times10^{5}$	+ (3)	+ (1)	0	0
	SC b	+ +	$8\ 0\times10^{3}$	+ (2)	+ (1)	0	0
9	N–a	—	$2\ 1\times10^{7}$	0	0	0	0
	SC–a	+ +	$4\ 1\times10^{6}$	+ (9)	+ (2)	Keratoconjunctivitis	0

* In each calf one of the eyes was selectively irradiated with ultraviolet light for 10 min the day before and again immediately prior to the inoculation (10) Both eyes were inoculated with the same bacterial culture The inocula indicated in column 4 consisted of 0 2 ml of overnight broth cultures The conjunctival flora was thereafter examined daily by swabbing and culture until *M bovis* could no longer be recovered (0 in columns 5 and 6 indicates that the organism could not be reisolated 1 day after inoculation and during the following 2–3 days) Three daily cultural controls prior to infection were all negative with respect to *M bovis*

§ Colony morphology on human blood agar (3), SC strongly corroding with zones of spreading and corroding growth of varying width, N non spreading, non-corroding

† All cell lines employed have been included in previous studies on morphology and competence in genetic transformation (3, 4) Some of the cell lines have also been examined for twitching motility, under identical designations (8)

* + + strongly fimbriated, (—) fimbriae occasionally observed in the preparations — no fimbriae detected (Fig 5 and ref 3)

blood agar, some differences were noted in two of the strains when the cell lines were again grown on bovine blood agar as a control, just prior to the infection experiments This was the case with the SC variants of strains 3 and 5 After at least 15 passages on human blood agar with up to 2 weeks interval they now formed convex colonies (although they were still corroding) on bovine blood agar medium, with various degrees of spreading Such a changed colony morphology on bovine blood agar was not found after passages of the other SC cell lines on human blood agar Thus, the culture of 4 SC used for infection was found to yield flat colonies on bovine blood agar after about 20 passages on human blood agar The cell line 9 SC originated from a diseased calf in a previous series of experimental infection The primary culture consisted exclusively of the flat colony type After 5 passages on human blood agar plates at 33° C it still formed typical colonies on the bovine medium, sometimes together with a few convex (non corroding) variant colonies

A mutual correspondence between the con

Fig 5 *M bovis* 9, primary culture from diseased bovine eye, same material as in Fig 2 The electron microscopical preparation was made after colony photography The colony material was removed with a sterile spatula and spread on a glass slide before suspension in 0 4 per cent sodium silicotungstate (pH 6 5) by pipetting A small drop of the light greyish suspension was transferred to a carbon

Cell wall extensions (double arrow) and units probably derived from these (triangle), showing fine structure are also evident (7)

5

vex (smooth), non corroding colony type on bovine blood agar and the N type on human blood agar (3) was found

The number of passages on human blood agar before use in the infection experiments was close to identical for each pair of N and SC type cell lines belonging to one and the same strain (Table 1) In strain 9, the N cell line used originated as a variant in the first transfer from a primary culture colony like the one shown in Fig 2, from which also originated the SC cell line used The two cultures were passaged identically on human blood agar, 5 times before infection

As controls, the bacterial cultures used for infection were streaked on bovine blood agar immediately prior to the inoculation The colonies from the 3 SC a and 5 SC b cell lines were exclusively of the convex, corroding type In the 9 SC a culture, about 5 per cent of the colonies were of the non corroding type In the 4 SC a and 5 SC a inocula, about 10 per cent of the colonies were non-corroding The cultures of N cell lines used for inoculation without exception gave typical non corroding colonies Electron microscopical preparations from the SC cell lines revealed strong fimbriation of the cells The N cell lines were non fimbriated (in one case a single fimbria was occasionally detected) (Table 1 and ref 3)

The infection experiments (Table 1) showed that only SC cell lines of *M bovis* were established on the mucous membranes of the eye Not a single non corroding colony was detected in the primary cultures even when the inoculum contained such colony variants (see above) Reisolation of the organism after infection with N cell lines was not possible in any case The characteristics of *M bovis* colonies on the primary plates (bovine blood agar) were the same as in the cultures used for infection except for the absence of non corroding variants Thus the 4 SC and 9 SC colonies were flat and corroding and the 3 SC and 5 SC colonies convex and corroding (see above) In each case where *M bovis* could be recovered from an infected eye, one or several consecutive isolates (usually each daily isolate) were subject to electron microscopy by negative staining Without exception the bacteria were fimbriated as the ones used for inoculation (Fig 5)

In no instance was a non haemolytic *M bovis* variant observed In most cases, a diversity of microbes in small numbers were detected similarly in both eyes before and after infection (staphylococci, non haemolytic streptococci, corynebacteria and Gram negative rods other than *M bovis*) In the calf infected with *M bovis* 9 SC a, a population of *Neisseria ovis* not present in the control cultures before inoculation was found in both eyes after *M bovis* infection *N ovis* persisted after the disappearance of *M bovis* Also in the *N ovis* isolates fimbriae were looked for and detected

Table 1 shows that ultraviolet irradiation of the eyes facilitates the initiation of *M bovis* colonization, just as the colonization is of a longer duration in irradiated than in non irradiated eyes if established

Only the calves infected with the fimbriated variants of the strains 4 and 9 developed disease In both of these animals only the eye that had been irradiated before inoculation showed clinical signs No disease occurred in any of the other infected animals The irradiation alone provoked only mild erythema of the skin around the eye and a slight increase in the moisture of the conjunctiva

The calf infected with the 4 SC cell line developed marked conjunctivitis of the irradiated eye The period of incubation was 24 hr The conjunctival inflammation began to decrease 2 days after the infection and the eye was normal on the 4th day after infection The calf infected with the 9 SC cell line developed a mild case of keratoconjunctivitis After an incubation period of 36 hours, the animal showed marked serous lachrymation increased blinking and photophobia The conjunctival mucosa and the third eyelid were swollen and hyperaemic In the centre of cornea there was a 3–4 mm circular opacity The corneal changes did not develop further the following days and on the 6th day after infection the conjunctival inflammation had

disappeared The cornea was clear and normal in appearance 35 days after the infection

DISCUSSION

The mechanisms by which bacteria, both innocuous parasites and pathogens, are established on mucosal membranes are to a large extent unknown Some bacteria show a high degree of selectivity in only colonizing special mucosal areas (see ref 18) The first prerequisite for bacterial colonization is attachment of the organism to the mucosal epithelium (18) The attachment may be mediated through particular surface properties of the bacteria (18) The findings in the present investigation that exclusively fimbriated variants (SC) of *M bovis* were able to colonize the mucous membranes of the bovine eye indicate the possible role of fimbriation as an adherence promoting factor It is known that fimbriae confer adhesive properties of bacteria to many kinds of tissue cells (5)

In the surface to surface interaction between the bacterium and the mucosal epithelium also the conditions of the mucous membrane itself may influence the attachment. In the present study it was found that the colonization was more readily established and of longer duration in eyes irradiated by ultraviolet light It is possible, therefore, that the tissue injury provoked by the ultraviolet light facilitates attachment of *M bovis* It has previously been shown that ultraviolet light enhances the pathological effect of *M bovis* infection on the bovine eye (9 17)

Although apparently closely associated with the ability to colonize the bovine conjunctiva, the occurrence of fimbriae and corroding colonies in *M bovis* does not seem to be a sufficient requirement for the organism to be virulent The ultimate virulence factor seems to be more closely associated with certain details in colony morphology, detectable in some of the strongly fimbriated variants on bovine blood agar medium Hence, the colony morphology in primary cultures from spontaneously as well as from experimentally diseased animals has been of the flat type

In some other reports on infectious bovine keratoconjunctivitis a smooth colony type of *M bovis* has been stated to be positively related to disease promoting ability, as previously discussed (16) In one study, however, rough colony forms of this organism were isolated from diseased eyes and shown to dissociate rapidly to a smooth colony form in subculture (1) Of considerable interest are the findings by *Jackson* (11) that the smooth, virulent colony type of *Haemophilus (M) bovis* isolated from infectious bovine keratoconjunctivitis was characterized by having a firm breakable consistency and leaving a small depression in solid medium when removed from the surface In subculture it showed variation to a rough, avirulent colony type which was easily emulsifiable and gave no depression in solid medium Since our rough, flat type of colony is friable (16) and corroding, it is probable that this type does, in fact, correspond to the virulent type named smooth by most other authors The exact cultural conditions appear to be very critical for the expression of a certain colony type, as also shown in the present study

In *Neisseria gonorrhoeae*, a correlation between colony morphology and virulence has been described (12, 14, 15) Recently, it was demonstrated that only cells from virulent colony types are fimbriated and it was suggested that the fimbriae may alter the host parasite relationship, possibly by hindering phagocytosis or by promoting adherence of the organism to epithelial cells (13 19) *N gonorrhoeae* is the only species other than *M bovis* in which it has been possible to show a distinct positive relationship between fimbriation and colonization/virulence This was generally not found for type-1 fimbriation in enterobacteria (5)

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NEISSERIA ELONGATA PRESENTATION OF NEW ISOLATES

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Thirteen strains, or clones, of oxidase positive, rodshaped bacteria from the human pharynx and a perimandibular abscess were identified as *Neisseria elongata* by comparison with the type strain of this species in conventional cultural and biochemical tests and in genetic transformation The study establishes *N elongata* as an entity of more than sporadic occurrence in pharyngeal cultures

In a previous report (9), a new rodshaped, oxidase positive bacterial species, with the proposed name *Neisseria elongata*, was described Determination of affinities in genetic transformation and of DNA base composition (9) revealed no compatibility with genus *Moraxella*, but a close relationship to members of the coccal genus *Neisseria* ("true neisseriae") Later studies by pulse-RNA to DNA hybridization (4) and gas chromatography (10) verified this pattern of affinities

Since only one isolate, the type strain M 2 (= ATCC 25295, NCTC 10660), existed and could be included in the previous studies, and since the observation is of general importance for the evaluation of cellular shape as a criterion in bacterial classification, it was of interest to search for and study more strains of this species The present report concerns additional isolates, compared with the type strain by conventional procedures and in genetic transformation tests

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MATERIALS AND METHODS

Bacterial Strains

Including the type strain of *N elongata*, the material consisted of 14 strains or clones (collectively called strains in the following) isolated from 9 clinical specimens. The isolation and properties of the type strain, M 2 (= ATCC 25295, NCTC 10660), has been reported (9) Two strains, 7823/71 I and II, were isolated from a culture of pus from a perimandibular abscess, and 11 strains were isolated from 7 cultures from pharyngeal swabs The designation of these strains are 8554/71, 9043/71, 11953/71, 516/72, 1488/72 I, II and III 1503/72, and 1737/72 I, II and III Each strain was derived from a single colony in the primary culture

Isolation Procedure

Pharyngeal swab cultures on blood agar medium (see below) were scrutinized with a magnifying glass, and from 3 to 5—most often 4—different *Neisseria*- or *Moraxella* like colonies were picked with a loop and subcultured on plates of the same medium Care was taken to select colonies of different appearance when possible After over night incubation Gram stained films were examined, and those subcultures which contained small Gram negative rods and were oxidase positive were purified by repeated plating from isolated colonies As colonies of *N elongata* do not appear to differ markedly from other pharyngeal organisms—at least not when seen in primary cultures—, the

search for *N elongata* could only be done in a haphazard manner, and it was not feasible to establish clear criteria for the selection of the right type of colony

Morphological Cultural and Biochemical Tests

The methods were mainly the same as those employed previously (8, 9) Primary cultures were made on human blood agar medium (7) and colony characteristics studied after incubation at 33 35° C in a humid atmosphere for 1–2 days (primary cultures) or more

Transformation Methods

The principles of mutant selection, preparation of transforming DNA and transformation techniques, have been described previously (2 3, 9)

RESULTS

Occurrence and Relation to Disease

The strains 7823/71 I and II were found in a culture of pus from a perimandibular abscess suspected of being actinomycotic The sample was too small for the demonstration of actinomycotic "sulphur granules" and only a single sample was received, so the etiology of the abscess is unknown The aerobic primary culture contained a number of low convex greyish colonies of clay-like consistency consisting of small Gram negative rods and giving a positive oxidase reaction (dimethyl p phenylenediamine reagent) Two separate colonies, which differed slightly in appearance were subcultured and purified The culture also contained viridans streptococci

The detection of these strains was followed by a systematic search for other strains of this organism in the pharynx Cultures from 461 outpatients in Oslo were studied as described under Materials and Methods, and a total of 11 additional strains were isolated from 7 different cultures i e, an incidence of positive cultures about 1 5 per cent This suggests that *N elongata* may not be a very common inhabitant in the pharynx at least not in sufficient numbers to constitute the majority of *Neisseria* like colonies

It frequently appeared that all or at least the majority of *Neisseria* like colonies belonged to a single species In many cases this turned out to be the oxidase negative *Micrococcus mucilaginosus* (1), in others oxidase positive colonies of identical appearance, probably representing only one coccal *Neisseria* species In one culture (1488/72) three out of three colonies examined were *N. elongata*, and in another case (1737/72) three out of four colonies Transformation results (see below) indicate that these colonies in each case represented a homogeneous population

In the course of the study an apparently antagonistic effect of viridans streptococci on *N elongata* was observed Whether this phenomenon, which is dealt with in a separate communication, could have influenced the isolation frequency of *N elongata* from pharyngeal cultures, is unknown

There is no strong indication that any of the strains was acting as a pathogen This possibility cannot be excluded, however The clinical diagnoses in most cases were trivial ailments such as asthma, allergic rhinitis, polyposis nasi, rhinopharyngitis or chronic tonsillitis

Morphological, Cultural and Biochemical Characteristics

The results of conventional diagnostic tests including determination of antibiotic sensitivity, were generally as reported for the strain M 2 (9) A few points deserve mention, however

The colonies on blood agar were low convex or nearly flat, glistening and greyish with a slight yellowish tinge On prolonged incubation separate colonies increased in size up to 4 5 mm diameter When the colonies were removed marked pitting of the agar surface was regularly seen After growth in a moist atmosphere for a few days, some of the colonies often became surrounded by thin granular spreading zones similar to those reported for fimbriated variants of *Moraxella nonliquefaciens* (6, 7, 15), *M bovis* (7) and *M kingii* (12, 13), or the colonies became irregular with more or less dentate

margins A detailed examination of colony type variation was not undertaken in this study, neither was electron microscopy performed The claylike consistency of the colonies was highly characteristic When colonies were subcultured on to new plates, it was practically impossible to spread them out evenly, as they formed lumps of dry particles on the surface It was impossible to suspend them evenly in water or saline, and they appeared to be highly hydrophobic

The catalase reaction was always weak or absent, but sometimes it became distinct when the liberated oxygen was trapped by placing a microscopic cover glass over the colonies

Genetic Relations

The results of genetic transformation experiments with streptomycin resistance as the marker, are summarized in Table 1

When the type strain, M 2, was used as recipient, all the other strains showed a ratio of interstrain to autologous transformation higher than 0 01 In this series, the strains 8554/71 9043/71, 11953/71 and 516/72 had ratios above 0 1 Among those with least affinity to M 2 the strains 1488/72 I, II and III showed a very high degree of genetic compatibility with each other (ratios of interstrain to autologous transformation equal to or higher than 0 5) A similar high degree of interrelationship was observed between the strains 1737/72 I, II and III When the strain 1737/72 I was used as recipient, 1488/72 III showed a ratio of interstrain to *autologous transformation about* 0 3 Thus the six strains 1488/72 I, II and III and 1737/72 I, II and III (probably representing only two different populations) may be considered as one entity The two remaining isolates examined (7823/71 I and 1503/72) had a somewhat reduced affinity also to this group of strains

When the strain 8554/71 was exposed without DNase termination (continuous DNA exposure (3)) to transforming DNA's from *N meningitidis*, *N flava* and *N flavescens*, numerous (250–1500 per plate) transformants were observed although distinctly less than with DNA from *N elongata* M 2 DNA from streptomycin resistant mutants of *N catarrhalis*, *Moraxella nonliquefaciens* and *M kingii* had no transforming effect

TABLE 1 *Ratios of Interstrain to Autologous Transformation among* Neisseria elongata *Strains**

	Recipient strain		
	M2	1488/72 III	1737/72 I
Donor strain			
8554/71	3.1×10^{-1}		
9043/71	1.5×10^{-1}		
11953/71	4.6×10^{-1}		
516/72	7.3×10^{-1}		
1488/72 I	2.2×10^{-2}	4.9×10^{-1}	
1488/72 II	2.1×10^{-2}	8.9×10^{-1}	
1488/72 III	2.3×10^{-2}		3.2×10^{-1}
1737/72 I	2.4×10^{-2}		
1737/72 II	2.4×10^{-2}		8.8×10^{-1}
1737/72 III	2.1×10		7.6×10^{-1}
7823/71 I	2.6×10^{-2}	7.8×10^{-2}	6.4×10^{-2}
1503/72	1.7×10^{-2}		7.9×10^{-2}
M2		4.8×10^{-2}	4.4×10^{-2}

* Quantitative streptomycin resistance transformation was used as previously described (2 3, 9), with few modifications In each experiment identical samples of the recipient (10^7 to 10^8 cells/ml) were exposed simultaneously to the respective donor DNA samples (20 μg/ml) Following DNA exposure for 45 min at 33° C DNase was added One tenth ml aliquots of appropriate dilutions were spread in duplicate on blood agar plates The plates were incubated for about 7 hr Then streptomycin was added from below the agar, followed by continued incubation for 3 to 4 days before assay of transformants The donor mutants had been selected at 500 μg streptomycin/ml the transformants were assayed at 50 μg/ml Most often about 0 1 per cent of the recipient population was transformed in the autologous parallels (those with DNA from a streptomycin resistant mutant of the recipient) The number of transformants obtained with each donor DNA was divided by the autologous transformant yield to give the figures of the table

DISCUSSION

With the present study, we consider the species *Neisseria elongata* to be established as more than a peculiar and rare finding, al-

though not very common, and that the pharynx may constitute its main habitat. When looked for in the nasal cavity in a previous study, *N. elongata* was not detected (5)

The affinities in genetic transformation of the individual strains reveal some heterogeneity of the strain material, with at least three groups of strains with interstrain to autologous transformation ratios below 0 1 when comparing strains belonging to different groups. This opens the possibility that the groups are as taxonomically distant to each other as are some other entities of genus *Neisseria* that are, perhaps doubtfully, considered as separate species (11). However, it is known from other studies that the compatibility apparent in streptomycin resistance transformation may sometimes be of the same relatively low order between strains of one and the same species of *Neisseria*, e g, *N meningitidis* (*E Holten, K Bøvre* and *K Jyssum*, unpublished). For the time being therefore no strong argument can be put forward to subdivide our present isolates into different taxa. In any case, several of the strains show high genetic compatibility with the type strain M 2, similar to the interstrain compatibilities found between isolates within one and the same species of *Moraxella* (3). When tested for genetic compatibility only with the coccal species of the "true" genus *Neisseria* (14) was detected as expected from previous results with the strain M 2 (9)

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SEROLOGICAL GROUPING OF STAPHYLOCOCCAL PHAGES BY INDIRECT HAEMAGGLUTINATION

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Phages in the international basic set used for routine typing of *Staphylococcus aureus* were grouped serologically with the indirect haemagglutination inhibition test The results showed complete agreement with the serological division of these phages into 4 groups A, B, F and L, which had earlier been done by neutralizing tests 33 additional phages isolated from 29 *Staphylococcus aureus* strains of human source were investigated and found to belong to group A or B Phages of each group were antigenically identical and showed no crossreaction with phages of any other group

Neutralizing tests have shown that staphylococcal phages attacking pathogenic strains of human source can be devided into seven sero logical groups A, B, D, F, G, H and L (9, 10, 14) The majority of the phages examined belong to groups A, B, and F The phages used in the basic set for routine phage typing of staphylococci belong to one or another of these three groups with the exception of phage 187, which belongs to group L (2) Phages of the same serological group are homogeneous in morphology and buoyant density and differ in these respects from those of other serological groups (3, 12) It has also been shown that the serological grouping is correlated to calcium requirement, stability and ability to transduction (5, 10, 14, 15)

The action of neutralizing antibodies is confined to the tail of the phage, while other antigenic determinants of the phage capsid react with antiphage antibodies, which do not participate in the neutralizing process (4, 7)

This paper reports the results of serological classification of staphylococcal phages by indirect haemagglutination, i e by means of a system which measures the reaction between antibodies and antigenic determinants on the entire capsid of the phage The main purpose was to find out to what extent the results obtained with this system agree with those based on neutralizing tests

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MATERIALS AND METHODS

Phages

23 phages in the international basic set used for routine typing of staphylococci and 33 phages from *Staph aureus strains isolated from healthy nasal* carriers and from patients with staphylococcal in fections were investigated

Phages A1, 29 and 42D were used as test phages for adsorption to tanned red cells Phages 29 and 42D belong to the basic set of the typing phages Phage A1 was isolated in our laboratory It attacks mostly staphylococci of patterns 80 or 80/81 It produces distinct, easily counted plaques on the propagating strain Titration shows a linear rela tionship between the concentration of the phage suspension and the number of plaques A1 is there fore suitable for quantitative calculation of the number of plaques (8) Phages 29 and 42D

duce small plaques, which are difficult to count These phages are therefore less suitable for this purpose

Propagation, titration and harvesting of the phages were done by the agar layer method (1) Media Tryptone Soya Agar and Tryptone Soya Broth (Oxoid) On propagation and titration 400 µg $CaCl_2$/ml was added to the media The strain selected for the propagation of phage Al (P S Al) was of pattern 80/81 Phages 29 and 42D were propagated on P S 29 (NCTC No 8331) and on P S 42D (NCTC No 10033), respectively (2) The phage suspensions were sterilized by passage through a membrane filter with a mean pore size of 0 45 µm and stored at +4° C The phage preparations which were used for the production of antisera and for adsorption to erythrocytes were made by propagating one passage from a stock of phages prepared by propagating one passage from a single plaque

Purification of phage antigen for the production of antisera was done by gelfiltration on agarose columns (Sepharose 4B or 6B) Bed dimension 2 5×35 cm Eluant 0 05 M sodium phosphate buffer, pH 7 4 Flow rate 6 ml/cm² per hour About 50 per cent of the phages were recovered in void volume

The phages were concentrated by ultrafiltration A Diaflo membrane XM 100 was used The concentrated phages were then suspended in Tryptone Soya Broth

Antisera were prepared by a single deep subcutaneous injection into rabbits of 1 2 ml purified phages with a titre of 5×10^{9}–1×10^{10} plaque forming units (p f u)/ml suspended in Freund complete adjuvant The animals were bled 6–7 weeks after the injection

Preparation of Formalinized Erythrocytes

Blood from a selected sheep was collected in Alsever's solution and stored at +4° C for 10–12 days The erythrocytes were afterwards treated with formaldehyde, as described by *Weinbach* (16) The formalinized erythrocytes could be stored at +4° C for at least one year as a 10 per cent suspension in distilled water Merthiolate 1 10,000 was added as a preservative

The tannic acid treatment was performed according to the method described by *Wide* (17)

Adsorption of Phages to Tanned Erythrocytes

Tanned erythrocytes in phosphate buffered saline (PBS) were concentrated to about 20×10^{9} cells/ml by centrifugation The exact number was determined in a counting chamber Tanned erythrocytes were added to a final concentration of 0 5–1 0 × 10^{9} cells/ml to a suspension of phages usually 3–4 ml The phage erythrocyte mixture was incubated at the desired temperature and for the desired time and then quickly cooled to room temperature by dilution in cool (+4° C) broth whereafter the erythrocytes were spun down The cells were resuspended to a concentration of 1–2 per cent in 1 per cent formaldehyde in PBS, pH 6 4, and incubated at +45° C for 45 minutes The cells were then washed twice in PBS, pH 6 4, and resuspended to a concentration of 3 per cent The fixation of the adsorbed phages with formaldehyde permitted storage of the phage coated cells at +4° C for at least 6 months without loss of quality

A control tube with phage suspension was incubated at the same time as the phage erythrocyte mixture The fraction of heat-inactivated phage was determined by titration from the control tube The fraction of adsorbed phage was calculated by titration of the phages in the supernatant after centrifugation of the tube with the incubated phage erythrocyte mixture and comparison with the fraction of heat inactivated phage

Formalinized tanned erythrocytes treated in the same way as the phage coated erythrocytes but not exposed to phage served as control cells

Performance of the Indirect Haemagglutination Test (IHA)

Twofold serial dilutions of antiserum were prepared in PBS, pH 6 4, containing normal rabbit serum (NRS) 1 400 as a stabilizor 0 05 ml of the 3 per cent suspension of phage coated cells was added to 0 35 ml of the serum dilutions in round bottomed glass or plastic tubes (inner dimension 10 mm) The tube rack was shaken carefully and the reactions were read in the usual way with aid of a mirror after the tubes had been allowed to stand for 2–24 hours at room temperature

Antigen control Phage coated cells in PBS with NRS 1 400 were used Serum control Control cells were added to the antiserum dilutions

Reading of Patterns

- + an even mat of cells covering the bottom of the tube
- (+) an even mat of cells delineated by a thin ring
- (—) a narrow ring surrounding a mat of cells
- — a 'button' or a narrow ring of cells without a mat

The highest dilution of serum giving a + or (+) reaction was taken as the antibody titre

The patterns remained unchanged for 18–36 hours with the exception of strong positive reactions, when the margin of the mat of cells curled down after 2–3 hours and could erroneously be read as negative reactions

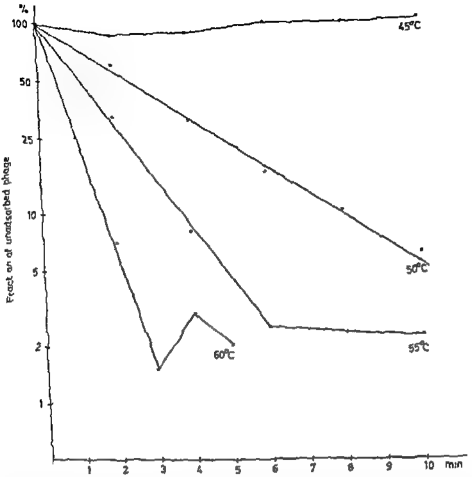

Fig 1 Influence of variation of temperature on adsorption of phage A1 to tanned sheep erythrocytes The cells were treated with tannic acid at +56° C in phosphate buffered saline, pH 7 2 Concentration of tannic acid 1 40 000 Incubation 30 min Adsorption with the phage-erythrocyte mixture suspended in Tryptone Soya Broth, pH 6 4 Number of cells 6×10^8/ml

Haemagglutination Inhibition Test (IHAI)

The end point (one agglutinating unit) of the titre of the antiserum was determined by 1 2 fold serial dilutions Two-fold serial dilutions of sterilized suspensions of phage were made in PBS pH 6 4 containing NRS 1 400 To each tube containing phage dilution (0 35 ml), antiserum (0 05 ml) was added to give a final titre of 5 10 agglutinating units The tubes were allowed to stand for 30 minutes at room temperature after which 0 05 ml phage-coated cells was added to each of them The reactions were read in the same way as the IHA The presence of broth in the tubes did not interfere with the reactions

Antigen control as in IHA *Positive control* Phage-coated cells and the dilution of antiserum (5 10 agglutinating units) without addition of phage

Tannic Acid Treatment

Influence of the concentration of tannic acid and pH on the adsorption of the test phages The experiments were designed on the basis of data given by *Wide* (17) as optimal for the adsorption of human chorionic gonadotrophin to tanned erythrocytes The time and the temperature for the incubation (30 min and +56° C rescpectively) and the concentration of cells (2 per cent) were kept constant The concentration of tannic acid was varied from 1 5 000 to 1 80 000 pH was varied from 5 6 to 7 8 The test phages were adsorbed (see below) to the different preparations of tanned cells and the fraction of adsorbed phage was determined The titres of the antiserum to the phage coated cells were tested by IHA The optimal concentration of tannic acid was 1 20 000 1 40 000 Concentrations of tannic acid

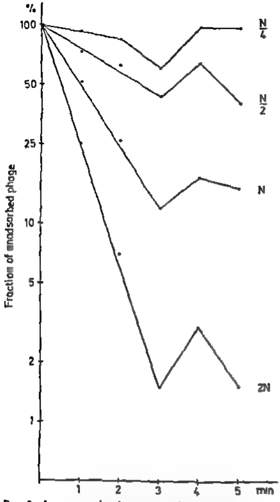

Fig 2 Kinetics of adsorption of phage A1 to tanned sheep erythrocytes of various concentration The cells were treated with tannic acid at +56°C in phosphate buffered saline pH 7 2 Concentration of tannic acid 1 40 000 Incubation 30 min

Adsorption with the phage erythrocyte mixture suspended in Tryptone Soya Broth pH 6 4 Temperature +60° C N ~ 3 × 10⁹ cells/ml

1 40 000 decreased the adsorption with falling titres in IHA as a result Concentrations above 1 20 000 did not result in an increased adsorption but tended to give indistinct patterns in IHA The optimal pH was found to be 7 2 At lower pH the adsorption decreased with falling titres in IHA as a result At pH higher than 7 2 the cells sometimes agglutinated spontaneously in IHA

Adsorption of Phage Antigen to Tanned Erythrocytes

Influence of suspension medium, temperature and pH on adsorption of the test phages No adsorption could be demonstrated when the phage erythrocyte mixture was suspended in PBS When broth was used as a suspension medium the phages were adsorbed to the cells provided that the temperature of incubation was at least +50° C (Fig 1) Phage 42D was absorbed best at pH 7 2, phage Al and 29 at pH 6 2 6 4 Purified phages suspended in broth were adsorbed less well and less regularly than phages in the original suspension Sterile filtered non purified phages suspended in broth were therefore used for coating of the cells The adsorption was usually done at +50° C for 10 minutes Under these conditions 95–98 per cent of the Al and 42D phages and about 60 per cent of the 29 phages were absorbed

The adsorption of phage Al followed a curve of first order kinetics for a certain period, after which *phages were eluted from the cells The elution was* temperature dependent and appeared earlier at higher temperature (Fig 1, Fig 2) The cause of the elution was not investigated It might be related to denaturation of one or several of the constituents of the broth which are necessary for adsorption of the phages to the erythrocytes Phages 29 and 42D were not examined in this respect because, mentioned above, they are less suitable for quantitative calculations

Specificity and Sensitivity of the Testing System

Table 1 gives the highest titres obtained in IHA with the antisera used and the cells coated with homologous antigen (TC Al TC 29 and TC-42D)

TABLE 1 *Agglutination with Antisera against the Staphylococcal Phages A1 29 and 42D of Tanned Sheep Erythrocytes Coated with the Phages Respectively*

	TC A1	TC 29	TC 42D	Uncoated cells
anti A1*	51200	<50	<50	<50
anti 29	100	12800	<50	<50
anti 42D	<50	<50	51200	<50

*Titres expressed as reciprocals of serum dilution

TABLE 2 *Inhibition of Agglutination of Erythrocytes Coated with Staphylococcal Phages A1, 29 and 42D in Test with 5–10 Agglutinating Units of Antiserum*

Inhibitor Phages	Titre of inhibitor (p f u $\times 10^9$/ml) In tube 1	Titre of inhibitor (p f u $\times 10^9$/ml) Giving inhibition	Serial twofold dilutions of inhibitor Tube 1	2	3	4	[illegible]	6
			Antiserum to phage A1 vs cells coated with A1					
A1	70	8	—	—	—	—	+	+
29	65	> 65	+	+	+	+	+	+
42D	100	>100	+	+	+	+	+	+
			Antiserum to phage 29 vs cells coated with 29					
A1	100	>100	+	+	+	+	+	+
29	50	3	—	—	—	—	—	+
42D	90	> 90	+	+	+	+	+	+
			Antiserum to phage 42D vs cells coated with 42D					
A1	100	>100	+	+	+	+	+	+
29	65	> 65	+	+	+	+	+	+
42D	50	3	—	—	—	—	—	+

— No agglutination (Inhibition)
\+ Agglutination (No inhibition)

and the titres with cells coated with heterologous antigen In IHAI with 5–10 agglutinating units the agglutination was inhibited in the homologous systems by phages with titres of 3–8$\times 10^9$ p f u /ml (Table 2) Phages to be examined in IHAI should therefore have a titre of 10–30$\times 10^9$ p f u /ml No inhibition was observed in the heterologous systems The highest titres of phages tested were 100$\times 10^9$ p f u /ml Attempts to increase the sensitivity by using a weaker system (2 3 agglutinating units) resulted in a reduced specificity with inhibition of the heterologous antisera

RESULTS

Grouping of the Typing Phages

All the typing phages with the exception of 187 could be demonstrated to belong to one of three groups as they clearly inhibited one of the three antisera but not any of the other two In the system used phages belonging to one of the groups were antigenically identical and they were antigenically different from phages belonging to either of the other two groups (Table 3) Groups A B and F, based on results with neutralizing tests, correspond ed to groups 1, 2 and 3, respectively In the IHAI as well as in the neutralizing test phage 187 (group L) proved antigenically different from phages of the other three groups (10) Thus, grouping of the typing phages by means of IHAI gave the same results as the neutralizing tests Hereinafter the groups will be referred to by the conventional letters

Grouping of Newly Isolated Phages

150 strains of *Staph aureus* were tested for production of phages with the method used by *Rosenblum and Dowell* (11) 60 *Staph aureus* strains including the propagating strains for the typing phages were used as indicator- and propagating strains

In the supernatants of broth cultures incubated at +37° C for 4–5 hours 59 (about 40 per cent) of the 150 examined donor strains showed phage activity Phages in 29 of the supernatants could be propagated to a satisfactory titre and sterilized by filtration without loss of titre Despite the use of several different sensitive strains little or no increase in the titres was found on propagating the phages in the remaining 30 supernatants with demonstrated phage activity The p f u in these preparations were too few to permit a grouping in IHAI

TABLE 3 *Grouping of 29 Staphylococcal Typing Phages by Indirect Haemagglutination Inhibition Test*

	Phage	Titre of phages (p f u × 10^9/ml) giving inhibition of 5–10 agglutinating units of antiserum*
Inhibition of anti A1 antiserum versus TC A1 Group 1	A1	8
	3A	10
	3C	20
	6	6
	42E	5
	47	6
	54	3
	75	1
	81	4
Inhibition of anti 29 antiserum versus TC-29 Group 2	29	3
	52	5
	52A	2
	79	2
	80	1
	55	8
	71	1
	53	1
	83A	8
	88	2
Inhibition of anti 42D antiserum versus TC-42D Group 3	42D	2
	77	6
	84	6
	85	12
No inhibition of the antisera used	187§	

* The titres of the phages tested were at least 8 16 times higher than those giving inhibition

§ Concentrated phage was used (titre 90 × 10^9 p f u /ml)

Phages in six of the 29 supernatants from which phages could be propagated to satisfactory titres were propagated on strains used for propagating the typing phages Three of these phages were propagated on P S 80 (NCTC No 9789) P S 47 (NCTC No 8325) and P S 83/83A (NCTC No 10039) respectively and three were propagated on P S 3A (NCTC No 8319)

Each of four of the 29 supernatants contained plaques of two different sizes Phages from a single plaque of each type were propagated on four strains Phages from the same supernatant were propagated on the same strain

All together, 33 newly isolated phages from 29 donor strains were examined in IHAI with 5–10 agglutinating units 31 of the preparations gave specific reactions, i e inhibition of one of the antisera used but not of the other two The phages propagated on P.S 80, P S 47 and P.S 83/83A, respectively did not belong to the same group as the corresponding typing phages Of the three phages propagated on P.S 3A, two were of the same serological goup as phage 3A (group A) and the third of group B The phages that were isolated in the supernatant from the same donor strain and could be separated on the basis of differences in plaque morphology belonged to different serological groups (Table 4)

TABLE 4 *Serological Groups of Phages Propagated on the Same Bacterial Strains*

Phages	Propagating strains	Serological groups
80	9789*	B
2672		A
3A	8319*	A
6802		B
37266		A
8976		A
47	8325*	A
14688		B
83A	10039*	B
8498		A
8497 1§	8630	A
8497 2		B
7667 1§	8023	A
7667 2		B
7933 1§	10081	A
7933 2		B
10506 1§	15736	A
10506–2		B

* = NCTC numbers

§ = Two phages of different plaque morphology were found in the supernatant from the same donor strain

Though they contained phages in a titre of about 3×10^{10} p f u /ml two of the phage preparations did not inhibit any of the anti sera Inhibition could have been expected if the phages belonged to group A B or F Antiserum prepared against these two phages gave agglutination of TC A1 (titre 1/51200) but not of TC 29 or TC-42D (titre <1/100) In IHAI where these antisera were used instead of anti A1 serum it was shown that these two phages were antigenically identical with the group A phages, and like the other phages of this group differed antigenically from phages of the other two groups

Of the 33 newly isolated phages 21 belonged to group A and 12 to group B

DISCUSSION

Specificity of the Testing System

The antisera used gave high titres in the indirect haemagglutination test (IHA) with the non purified homologous antigen and no measureable or negligable titres against the non purified heterologous antigens In the haemagglutination inhibition test (IHAI) with 5–10 agglutinating units of the antisera the agglutination was inhibited in the homologous but not in the heterologous systems (Table 1 and 2) The phage preparations used for the production of antisera were purified by gel filtration Thus the possibility had to be born in mind that the reactions between the antisera and erythrocytes coated with non purified test phages expressed reactions between antibodies and antigenic determinants specific for each of the three bacterial strains used for propagating the test phages This is however unlikely as several phages which were propagated on the same bacterial strain were of different serological groups (Table 4) Further the capacity of the phage preparations to inhibit in IHAI was correlated to the number of plaque forming units per ml Phages which lost activity during storage also lost capacity to inhibit the agglutination

The results of the serological grouping of the typing phages in the present investigation showed complete agreement with those obtained by other authors with neutralizing tests (2 10, 14) This does not mean that the antigenic sites which determine the specificity of each group in the neutralizing test are identical with those determining the grouping in the IHAI Neither does the striking specificity of the groups shown in the IHAI indicate that the capsid of the staphylococcal phage is a homogenous antigenic structure The capsid may contain several different antigenic determinants which are specific for the group It is thus conceivable that antigenic differences exist between the head and tail of the staphylococcal phage similar to what has been shown for the *E coli* phage T2 (7) It is probable, however, that the number of antigenic determinants of the capsid of the phage are limited since phage capsids, like most other viral capsids are built up of identical morphological subunits (3)

Only phages of group A or B were found among the newly isolated phages, but only about 50 per cent (29 out of 59) of the phages found in the supernatants from the broth cultures with the donor strains could be propagated to a titre high enough to permit examination in IHAI Like the group A- and B-phages the phages of group F have their source in lysogenic strains, but they are rather difficult to propagate (10) It is therefore reasonable to assume that a certain percentage of the phages that could not be propagated belonged to group F That phages of groups D, G, H and L were not found was expected Phage 187 is the only known representative of group L and the group G D- and H phages seem unable to establish lysogenic systems (10) Of the 56 phages grouped by IHAI none had demonstrable antigenic determinants in common with phages of another serological goup Thus no [illegible] found suggesting recombination of [illegible] different serological groups of [illegible] termining the synthesis of the [illegible] tures

The neutralizing test and [illegible] haemagglutination test are [illegible]

different types of serological reactions The former reveals antibodies directed against the tail of the capsid, the latter those directed against antigenic determinants located over the entire capsid There are also differences which may be of practical interest If phage coated erythrocytes, which can be stored for several months, are available, grouping of a large number of phages by IHAI is less laborious than by neutralizing tests

The results in the present investigation showed that a system weaker than 5 10 agglutinating units in IHAI could not be used without loss of specificity This means that the phages to be examined should have a titre of about 1×10^{10} p f u /ml Neutralizing tests do not require such high phage titres

It is well known that many staphylococcal strains are lysogenic and produce phages on growth *in vitro* The frequency of such strains has been given as 40–90 per cent (6, 11 13) In the present investigation phages were demonstrated in the supernatant of broth cultures of 40 per cent of 150 strains examined, despite the use of only 60 indicator strains Lysogenic staphylococci may produce phages during growth in a host organism An individual with a staphylococcal infection will presumably develop antibodies against the phages produced by the infecting strain If phage coated erythrocytes are available the indirect haemagglutination test would be a simple and rapid method for detecting antiphage antibodies in contrast to the laborious neutralizing test

It is therefore conceivable that the indirect haemagglutination test in which the antigen consists of purified staphylococcal phage may be of value in the diagnosis of staphylococcal infections and in the evaluation of the activity of such infections

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BRIEF REPORTS

FREUND S ADJUVANTS THEIR INFLUENCE ON THE SPECIFICITY OF VIRAL ANTISERA

P E Vestergård Jørgensen

A number of factors seem to influence the antibody production in experimental animals inoculated with viruses At least one factor is known to influence the cross reactivity of the resulting antibodies namely the number of virus inoculations given to the animal Accordingly as an animal is given 5 to 7 or 1 to 2 injections of say an arbovirus the resulting antiserum will be strongly or weakly cross reactive (*Casals* 1967)

The use of adjuvants in the production of viral anti sera is known to affect antibody production quantitatively i e, to lead to higher titres of antibody, and seems also to affect the durability of antibody production (*Kwapinski* 1965) Any information about the possible influence of adju vants on the cross reactivity of the antibodies pro duced has apparently not been published

It was reported recently (*Jørgensen* & *Grau balle* 1971) that two isolates of infectious pan creatic necrosis (IPN) virus which is a trout pathogenic reovirus (*Wolf et al* 1960 *Moss* & *Gravell* 1969) would cross react strongly if examined with one antiserum but not if examined with two other antisera It was suspected that this difference might be due to the fact that different adjuvants (i e Freund s complete and Freund s incomplete adjuvant respectively) had been used in the immunization procedure The purpose of the present investigation was to eluci date the possible influence of Freund s adjuvants on the cross reactivity of anti IPN sera

Material and Methods

Cell cultures RTG 2 cells, a cell line from rain bow trout gonads (*Wolf* & *Quimby* 1962), were grown as described elsewhere (*Jørgensen* 1972) at 20° C in glass bottles Nuclon plastic tubes and plastic Petri dishes (A/S Nunc Roskilde Den mark) Petri dishes were incubated in a 5 per cent CO_2 atmosphere during outgrowth After inocula tion with virus cultures were incubated at 15° C

Viruses IPN virus Strains Sp and Ab (*Jørgen sen* & *Grauballe* 1971) passage No 21 and No 11, respectively, were cloned by plaque selection be fore they were used for the production of inocula for rabbits Cell cultures were rinsed 3 times with serum free medium before they were infected with virus and the cells were maintained in serum free medium during virus multiplication As soon as strong CPE had appeared the cell culture fluid was centrifuged at 10000 g for 15 minutes to re move cellular debris The supernatant, containing approximately 10^{7} $TCID_{50}$ per ml was used for preparing the inocula

Experimental animals Twelve four month old rabbits were each given 3 inoculations of virus + adjuvant at intervals of 2 weeks A total inoculum of 4 ml was given each time Out of this 2 ml were given intramuscularly in a hind leg while 2 ml were given by way of five separate sub cutaneous injections on the back Three of the rabbits received Strain SP combined with Freund s complete adjuvant three others received the same [illegible] rabbits were bled [illegible] weeks after the third injection and sera from rabbits receiving the same virus-adjuvant combination were pooled prior to exami nation

Two rabbits were immunized in a similar man ner with extracts of normal RTG-2 cells One rab bit received complete adjuvant, the other receiving incomplete adjuvant

Serum inactivation The influence of the fol lowing treatments was examined Heat inactiva tion at 56° C for 20 minutes and treatment of sera with a homogenate of spleen kidney, liver and muscle from normal rainbow trout (under magne tic stirring over night at 4° C, followed by cen trifugation at 10000 g)

[illegible] incubated for one hour at 15° C Serum virus mixture in amounts of 0 2 ml, was then added to monolayers

Received 2 viii 72 from *State Veterinary Serum Laboratory, Dept for Jutland Hangøvej 2, 8200 Århus N Denmark*

TABLE 1 *50 Per Cent Plaque Neutralization Titres (Reciprocal Values) and Titre Ratios of Antisera*

Antiserum	Adjuvant used	titre Sp	titre Ab	titre ratio
Anti Sp	complete F	160 000	90 000	0 56
Anti Sp	incomplete F	51 000	4 000	0 078
Anti Ab	complete F	4 400	300 000	0 015
Anti Ab	incomplete F	350	300 000	0 0012
Anti RTG 2	complete F	< 10		
Anti RTG 2	incomplete F	< 10		

F — Freund s adjuvant

of RTG 2 cells in Petri dishes and the cell sheets covered with agar medium as described elsewhere (*Jorgensen* 1972) After 72 hours the plaques were developed by means of neutral red counted and the percentage of plaque neutralization observed at each serum dilution plotted on probability paper The 50 per cent neutralization titre was read by graphical interpolation At least 2 dishes were used per serum virus mixture

The serological relationship between the virus isolates 1/r was calculated from the formula $r = \sqrt{r_1 \times r_2}$ where r_1 and r_2 are the titre ratios (heterologous titre divided by homologous titre) for the respective antisera (*Archetti* & *Horsfall* 1950)

Results

As shown in Table 1 the viral antisera had high titres of homologous antibody whereas the anti RTG 2 sera in dilutions 1 10 did not neutralize Strain SP

Anti Sp serum produced with the aid of Freund s complete adjuvant had a higher titre of homologous antibody than the corresponding serum produced with the aid of incomplete adjuvant No such difference was observed for the anti Ab sera

Table 1 also shows that the heterologous titres of the antisera were highest if Freund s complete adjuvant had been used in the immunization procedure Table 2 shows this more clearly giving the calculated values for serological relationship 1/r for each of the two antiserum groups

Heat inactivation and absorption with tissue homogenate had the same effect on sera produced with either adjuvant the effect was in all cases less than a 2 fold titre reduction

Discussion

According to the *Committee on Enteroviruses* (1962) a new enterovirus isolate shall be considered antigenically distinct from a reference virus

TABLE 2 *Serological Relationship 1/r, between Strains Sp and Ab of IPN Virus*

Adjuvant used	1/r
Complete Freund s	10 8
Incomplete Freund s	103 0

if 20 units of antibody against the latter fail to neutralize 100 ID_{50} of the new isolate and vice versa In the formula of *Archetti and Horsfall* (1950), this corresponds to values of 1/r greater than 20 i e the virus isolate shall be considered antigenically distinct if 1/r is greater than 20

In the present investigation, two viruses SP and Ab were found to be serologically related in the above sense if examined by means of antisera produced with the aid of Freund s complete adjuvant (1/r = 10 8) whereas they were found to be serologically non releated (1/r = 103) if examined with sera produced with the aid of Freund s incomplete adjuvant

By electron microscopy the viruses have been found to be of the same size and gross morphology (*Block* & *Scherrer*, personal communication) Strain Ab seems to be less pathogenic than Strain Sp and does not as the latter produce CPE in FHM cells (*Jorgensen* & *Kehlet* 1971)

Such an effect of Freund s adjuvants on the specificity of antisera may probably be observed also if other viruses are involved The only difference between the adjuvants is that Freund s complete adjuvant contains killed tubercle bacteria in addition to the mineral oil which is the main constituent of both It is not known whether the complete adjuvant has caused the formation of cross-reactive antibodies by exposing viral antigens otherwise unexposed to the immune system of the animal or by inducing the production of another type of antibody

The question arises whether limits of serological relationship can be established without specifying

the characteristics of the antisera which are to be used in the examination

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FTA ABS TEST READING MADE EASY

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The FTA test (*Deacon et al* 1957) and its later modification the FTA ABS test (*Deacon et al* 1966, *Hunter* 1971) has greatly increased the diagnostic capacity for treponemal antibody determination in many routine serologic laboratories Reading the FTA ABS test in the fluorescent microscope is relatively easy but nevertheless requires a certain amount of experience Negative serum samples give no fluorescence which is obviously harder to document positively We have been using an 'internal reference" in a slight modification of the FTA ABS test which has made the reading easier To the *Treponema pallidum* suspension (BBL, Cockeysville, Maryland, USA) was added *Staphylococcus aureus*, strain Cowan I, treated with 0 5 per cent formaldehyde for 3 hours (*Lind* & *Mansa* 1968) to a concentration of 2×10^9 cells per ml This suspension, containing both *T pallidum* and *S aureus*, is then used for the preparation of the dried slide specimens Protein A of the staphylococcus shows a unique reactivity for the Fc part of normal gammaglobulin (*Forsgren* & *Sjöquist* 1966 *Kronvall* & *Williams* 1969, *Kronvall* & *Frommel* 1970) Since the *S aureus strain* used (Cowan I) contains large amounts of protein A immunoglobulin G from the patient will combine with the staphylococcal cell wall proteins via protein A reactive Fc structures Fluorescein labelled antihuman globulin will therefore always stain the gamma globulin coated staphylococci This adsorption of gamma globulin from the test sample has not been found to interfere with the specific staining of the treponemes as judged by parallel experiments with and without staphylococci The presence of stained staphylococci on the slides allows a rapid and positive indentification of the proper area to be examined in the microscope and also provides an internal reference of fluorescence

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muno lifusion Ac not detected in after adsorption n any of the sera of sera containing mononucleosis ally slightly reduced if adsorption at 45° C (which leads to complete poprotein as well as of the more l lipoprotein species (6)) The ass of antibodies is almost com by treatment with kaolin another l ipoprotein adsorbent (3)

ntigen positive sera were shaken at ambient temperature The antigen stimated by a counter electrophoresis modified according to *Alter et al* (1) est sera Au antigen titre ranging from no detectable antigen was present after in two of the sera the titre was reduced of between 8 and 16 The residual completely removed by a second Aero it Mixing during adsorption may have ineffi ient in this experiment in which dsorption of β lipoprotein was complete tigen particles may differ in their affinity to il and i n e a higher temperature or longer ation time may occasionally be necessary to n omplete adsorption as seen in the case of lipoprotein Shortage of materials prevented such t ting In one of these adsorbed sera electron microscopy disclosed Dane particles and 20 nm particles (8) in about the same relative proportion as that prior to adsorption, indicating that binding is not restricted to the small parti les In a second available serum containing Dane particles complete removal of antigen was readily achieved after a single batch adsorption Further evidence of a heterogeneity within the Au antige i particles with regard to affinity to Aerosil was obtained by column chromatography 80 ml of a serum containing Au antigen (titre 1 32) was applicated on a column (bed volume 20 ml made from 1 g of Aerosil) No antigen was released during application and washings with PBS During elution the NaCl concentration in 1 M *Tris* HCl buffer which was increased from 15 per cent to saturated solution (6) by 5 steps Au antigen was seen to be distributed in all fractions Hence a one step elution with saturated NaCl at pH 9 0 should be preferred for preparative purposes A considerable purification of Au antigen is achieved by this one-step procedure in which wide columns should be used to improve the flow rate accordingly as larger amounts of sera are handled

Lipid components on the surface of the antigen particle is probably the factor mainly responsible for its binding to Aerosil, since adsorbed Au antigen is partly eluted from Aerosil by 2 per cent Tween 80 and likewise, is poorly adsorbed in the presence of the detergent

Pretreatment with polyethylene glycol is known to reduce strongly several inorganic adsorbents affinity to protein (4) Adsorption of Au antigen was still nearly complete however, when Aerosil pretreated with 1 per cent Carbowax 6000 and washed was used in the batch procedure Furthermore adsorbed antigen is not released by treatment with 1 per cent Carbowax whereas free antigen is not adsorbed in the presence of this reagent

A solid phase radioimmuno assay based on this observation is under development in our laboratory Au antigen from patients' sera is fixed on Aerosil thus making a support for the binding of antibodies and subsequently for radiolabelled antigen in the presence of PEG

The hepatitis associated Au/SH antigen is possibly a part of the infectious particle If so the hepatitis hazards associated with serum transfusion may be greatly reduced by Aerosil treatment without involving β propionolactone (6) which strongly modifies the serum proteins

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